ANTI-INFLAMMATORY AND CYTOTOXIC ACTIVITIES OF MANGO (*MANGIFERA INDICA* L. VAR KEITT) POLYPHENOLS IN CANCER AND NON-CANCER BREAST FIBROBLASTS IN VITRO

A Thesis

by

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ABSTRACT

Breast cancer is the leading cause of cancer death among women worldwide and polyphenols are under investigation as an alternative to conventional treatment approaches of breast cancer. The anti-inflammatory and anti-proliferative activities of polyphenols have been demonstrated in many studies, yet cellular targets and the underlying cellular mechanisms remain unclear.

The overall goal of this study was to investigate the anti-inflammatory and cytotoxic properties of polyphenol compounds extracted from the mango variety Keitt in MCF-12A breast non-cancer and MDA-MB231 breast cancer cells by assessing the modulation of signaling pathways involved in inflammation and carcinogenesis. Mango polyphenols were identified by HPLC-MS analysis. The generation of reactive oxygen species was performed using fluorescence intensity in the DCFH-DA assay. Gene expression was analyzed by qRT-PCR, and protein expression was conducted by Western Blotting and Multiplex Bead assay analysis.

Bioactive compounds identified in the mango pulp by HPLC-MS included a great variety of polyphenols such as gallic acid, galloyl glucosides with different degree of polymerization and other polyphenols. The anti-inflammatory activities of mango polyphenols were evaluated in MCF-12A non cancer breast fibroblasts. An inflammatory microenvironment for MCF-12A breast cells was induced with tumor necrosis factor alpha (TNF-α). The generation of reactive oxygen species was suppressed significantly compared to cells induced with TNF-α, where there was no

significant difference between the concentrations of mango polyphenol extract. Results showed a significant down-regulation of mRNA and protein expression of inflammatory genes involved in the PI3K/AKT pathway and related downstream targets such as NF-κB and mTOR involved in biological processes including cell growth, proliferation and survival. Moreover, mango polyphenols had a significant impact on the miRNA-126-PI3K/AKT axis which plays an important role in inflammation and carcinogenesis, suggesting a potential anti-inflammatory underlying mechanism.

The cytotoxic effects of mango polyphenols were investigated in MDA-MB231 breast cancer cells. Mango polyphenols decreased the production of reactive oxygen species; however no significant differences were found between the tested concentrations of mango polyphenols. The gene expression of proapoptotic factors involved in the intrinsic mitochondrial pathway such as cytochrome C and caspase-3 were significantly regulated after mango polyphenol treatment. In addition, the suppression of the PI3K/AKT/mTOR pathway and downstream effectors such as HIF-1α and VEGF as well as the disruption of the miRNA-21-PTEN/AKT axis were identified as potential underlying mechanism of the cytotoxic properties of mango polyphenols.

Overall, findings from this study show that mango polyphenols counteract inflammatory and cancerous cell signaling processes; therefore the potential of mango polyphenols in the prevention of breast-cancer focusing on the PI3K/AKT/mTOR-axis should be further investigated.

DEDICATION

To my mom and dad

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CHAPTER I

INTRODUCTION

Fruits and vegetables are considered functional foods based on the presence of bioactive compounds that may be capable of preventing chronic-degenerative diseases as well as counteracting the detrimental effects associated with these. Several studies investigated novel sources rich in polyphenols that could be used as therapeutic or preventive agents. Among chronic diseases, cancer and specifically breast cancer is the leading cause of cancer death among women worldwide (*1, 2*). While polyphenolics show a high potential in the prevention of breast cancer in several studies, the underlying mechanisms and cellular targets of polyphenolics have not conclusively been investigated.

Chronic inflammation has been linked to several steps involved in carcinogenesis. Inflammation will influence the micro environment of cells and promote the generation of mutated cells (*3-5*). During inflammatory and carcinogenic processes a wide variety of signaling pathways are generally under or overexpressed and this may cause dysregulation of the cell cycle and proliferation. Dysregulated pathways constitute potential targets for chemopreventive agents. In addition, small non-coding RNAs or micro-RNAs have arisen as novel players in anti-inflammatory therapeutic strategies but the role of polyphenols in the regulation of miRNAs still remains unclear.

Mango (*Mangifera indica* Linn.) is a tropical fruit characterized by intense aroma, tasty flavor and high nutritional value. Specifically, mangos are rich in vitamin A and C, fiber and minerals (*6, 7*). In addition, mango contains moderately high concentrations of polyphenols which have shown antioxidant activity capable of counteracting the deleterious effects of free radicals involved in degenerative diseases such as cancer. In fact, experimental data has shown that bioactive compounds in mangoes may exert anti-inflammatory, anti-carcinogenic, antiviral and antibacterial properties in several *in vitro* and *in vivo* models (*6, 7*). Several studies have reported the anti-proliferative effects of mango polyphenols in breast cancer *in vitro* and *in vivo*; however their capacity to attenuate cancer-related inflammation as well as their potential ability to regulate small non-coding RNAs or microRNAs has not been elucidated yet, therefore, the objectives of this study are:

- To evaluate the anti-inflammatory activity of mango Keitt polyphenol extract in MCF-12A non cancer breast cells through the modulation of the PI3K/AKT signaling pathway and related downstream genes such as NF-κB and mTOR and to evaluate the involvement of miRNA-126 as a potential underlying mechanism.
- To investigate the efficacy of mango Keitt polyphenol extract as a cytotoxic agent in MDA-MB231 breast cancer cells by the modulation of apoptotic genes and the suppression of the PI3K/AKT/mTOR signaling pathway. The potential involvement of the miRNA-21-PTEN axis as an underlying mechanism of the anti-carcinogenic effects of mango Keitt polyphenols is also investigated.

CHAPTER II

LITERATURE REVIEW

Polyphenols

Polyphenols are secondary metabolites produced by plants and these are major bioactives of our diet, that can be found mainly in fruits, vegetables, seeds and products of these.(*8*) . Plant polyphenols are chemical compounds, produced in small amounts, which are not essential for regular plant growth, nutrition or development; however they are functionally involved in several structures and systems such as cell wall, protection against environmental, pathogens and plant injury. Most polyphenols in plants have glycosides with varying sugar units and acylated sugars covalently bound to different positions of the polyphenol skeletons (*9*). The amount and presence of polyphenols are influenced by various factors such as: variety, climate, harvest time, processing and storage (*10*).

Classification of polyphenols

Polyphenols are chemically categorized as compounds with phenolic structural features (*9*); however, they are fairly diverse and comprise several groups of phenolic compounds. Phenolic compounds are synthetized within three main metabolic pathways: cinammic, isoprenoid and shikimik acid pathway producing flavonoids, carotenoids and phenolic acids, among other compounds, respectively (*10, 11*). More than 8000 phenolic compounds have been described (*10*) and these can be divided into 4 different general

classes based on their chemical structure: phenolic acids, flavonoids, stilbenes and coumarines (*10, 12*). The chemical structures of these compounds are shown in Figure 1.

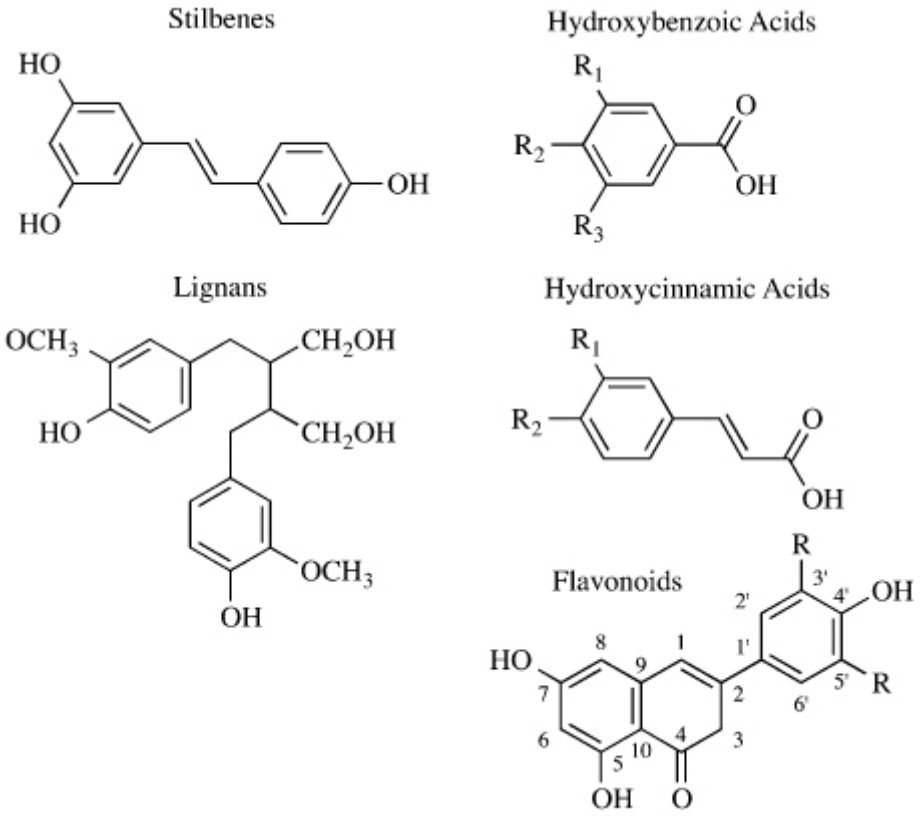

Figure 1. Chemical structure of phenolic compounds (*13*)

Phenolic acids

The structure of phenolic acids is characterized by one carboxylic acid functional group and two constitutive carbon structures, the hydroxycinnamic and hydroxybenzoic structures which are generally substituted with one or more hydroxyl groups (*14*). This group of compounds is generally present in a free form in fruits and vegetables; whereas

in grains and seeds they are often in a bound form which can only be freed or hydrolyzed by acid or alkaline hydrolysis, or enzymes (*9*).

Flavonoids

Flavonoids are ubiquitous in plants and are the most common and widely distributed group of polyphenols responsible for the pigmentation of flowers and leaves (*9*). The general structure of flavonoids consists of a flavan nucleus consisting of 15 carbon atoms arranged in a C6-C3-C6 ring in which the two C6 units are of phenolic nature (*13*). Almost 5,000 flavonoids have been identified and classified into at least 10 chemical groups (*15*). Among them, flavonols, flavanones, flavones, flavan-3-ols and anthocyanidins are the most predominant flavonoids identified and described (*13, 15*). The differences between these compounds are due to the hydroxylation pattern and variations in the chromane ring (*9*).

Lignans

This class of polyphenols is of great interest based on their antioxidant and anticarcinogenic activities (*16*). Lignans are secondary plant metabolites derived from the shikimic acid pathway which belong to the group of diphenolic compounds derived from the combination of two phenylpropanoid C6-C3 units at the β and β′ carbon atoms (*13, 17*). The main sources of dietary lignans are oilseeds (flax, soy and sesame), whole-grain cereals (wheat, oats, barley), legumes, vegetables and fruits, specially berries (*17*). The biological activity of lignans has been investigated in several studies and data has

shown the cytotoxic effects of lignans in several types of cancer (*16*); however, it has been suggested that not only lignans present in foods are responsible for promising anticarcinogenic effects but also other compounds or a combined action may contribute to the biological and functional properties of lignans (*16, 17*).

Stilbenes

Stilbenes compounds are synthetized from cinnamic acid derivatives and distributed in plants as monomeric dimeric, trimeric and polymeric stilbenes (*18*). The major stilbene identified and studied is resveratrol, a monomeric stilbene present in grapes and wines. *In vitro* and *in vivo* studies have highlighted its anti-inflammatory, anti-mutagenic and anti-cancer activity suggesting the use of resveratrol as a chemopreventive agent despite its fast degradation in most foods (*13, 18*).

Dietary Polyphenols and the Prevention of Diseases

The interest for plant polyphenols, especially those found in fruits and vegetables has been increasing in the last decade since they have been associated with a decrease incidence of chronic diseases such as cancer, cardiovascular abnormalities and inflammatory disorders (*8, 10, 11*). Even though the anti-inflammatory or anticancer activity of individual or single compounds have been shown *in vitro* and *in vivo*, the combination of a variety of polyphenols might increase their anticancer properties due to synergistic effects (*10, 11*). Experimental data obtained from cell culture and animal models as well as epidemiological studies have shown evidence that polyphenols present

in a diet rich in fruits and vegetables can reduce the incidence of stomach, lung, oral cavity, and colon and breast cancer (*12*). Such promising evidence provides strong support for the future acceptance of natural dietary compounds as either chemopreventive or therapeutic agents (*11, 12, 19*).

Chemoprevention with plant compounds has arisen as a promising approach or strategy to reverse, suppress, control or prevent the biological processes leading to the development of carcinogenesis or tumorigenesis using synthetic or natural agents such as phytochemicals and/or dietary polyphenols (*19*). The mechanisms by which polyphenols can be considered as chemopreventive agents are multiple and attributed mainly to their antioxidant properties, modulation of inflammatory pathways and induction of apoptosis (*11, 19, 20*).

Antioxidant activity

Polyphenols present in fruits and vegetables are well known for their antioxidant properties which are based on their redox capacity allowing them to act as reducing agents, hydrogen donators and singlet oxygen quenchers (*21*). Polyphenols are considered antioxidants agents if they fulfill two conditions. Firstly, polyphenols can delay or prevent the oxidation of a free radical when present in low concentrations and secondly, the radical formed after scavenging must be stable (*10, 21*). According to some authors, the most important factor explaining polyphenols' antioxidant activity is their chemical structure which allow them to trap, quench and/or scavenge reactive oxygen species (ROS), therefore preventing any reaction that could cause DNA damage

which in turns leads to carcinogenesis (*11*). ROS or free radicals include hydroxyl and peroxyl radicals, hydrogen peroxide and singlet oxygen, among others, are produced as a natural metabolism of oxygen in aerobic organisms (*20*) however an imbalance between their production and elimination (oxidative stress) has been reported to be harmful to cells. In addition, greater levels of ROS are responsible for the oxidative damage to cellular DNA, which in turn can lead to mutations indicating that ROS could be linked to cancer development (*22*).

Research has shown that antioxidant supplementation coming from polyphenols, in particular those found in fruits and vegetables can prevent and counteract ROS production (*11, 23*). In contrast, there is some evidence indicating that polyphenols can exert pro-oxidant activities, thus enhancing oxidative stress. According to some authors, the antioxidant or pro-oxidant activity of polyphenols is dependent on cell type and the concentration applied to them, as well as the level of oxidative stress within cells, that tends to be higher in cancer cells, conferring a paradoxical effect to polyphenols (*11, 20, 23*).

Modulation of inflammatory pathways

Inflammation is a natural response from the body to injure or infection caused by an internal or external factor; however when this process turns uncontrolled it may lead to several malignances including cancer. It has been reported that polyphenols play an important role in the modulation of inflammatory and anti-inflammatory pathways exerting diverse effects on several targets involved in these signaling pathways which

are related to cellular proliferation, apoptosis and inflammation, among others (*11, 19*). Pro-inflammatory cytokines, enzymes, chemokines and transcription factors are expressed during an acute or chronic inflammation process, among these; NF-κB (Nuclear factor kappa β) has been reported to be a key regulator of inflammation leading to the expression of several cytokines and enzymes such as TNF-α, COX-2, iNOS, HIF-1α, among others, which are involved in carcinogenesis development. *In vitro* and *in vivo* anti-inflammatory activity through the down-regulation of NF-κB has been attributed to polyphenol compounds (*20, 24, 25*) indicating that they exert multiple potential actions on inflammatory cascades related to chronic diseases.

Apoptosis

Programmed cell death or apoptosis is a complex process involving different signaling pathways (*19, 20*). Apoptosis is a genetically controlled form of cell death which is important for the normal embryonic development and for the maintenance of tissue homeostasis in the body; however, cancer cells are common to show an uncontrolled proliferation, and an inability to undergo programmed cell death (*26*).

Two apoptotic pathways have been described in mammalian cells: extrinsic (death receptor) and intrinsic (mitochondrial) pathways. The activation of both apoptotic pathways is carried out in different ways. The extrinsic pathway is activated when a specific ligand binds its corresponding cell-surface death receptor, like tumor necrosis factor (TNF) receptor, TNF-related apoptosis-inducing ligand (TRAIL) receptor and Fas receptor , whereas the intrinsic pathway is activated by diverse agents, such as oxidants,

drugs or ionizing radiations, all of which induce ROS overproduction leading to oxidative stress (*8, 11, 19, 26*).

Different members of the caspase family are involved in the activation of both pathways, for example caspase 8 and caspase 9 are reported to be responsible for the activation of the intrinsic and extrinsic apoptotic pathways respectively (*19*). Moreover, both apoptotic pathways lead to the activation of caspase-3 which, in turns, stimulates other executor caspases (*26*). The proteins of the Bcl-2 family control the major steps of programmed cell death. The regular maintenance or perturbation of mitochondrial membrane depends on the ratio between pro-apoptotic (Bax, Bad, Bak, Bid, Bcl-Xs) and anti-apoptotic (Bcl-2, Bcl-XL, Bag-1, Bcl-W) members of Bcl-2 family (*26*).

A great variety of polyphenols have been reported to induce apoptosis in cancer cells by modulating the expression of regulatory or controlling proteins such as cytochrome c with the subsequent activation of caspases-9 and caspases-3, down-regulation of Bcl-2 and up-regulation of Bax (*26-28*). Among the polyphenols screened and reported to induce apoptosis in cancer cells are resveratrol present in grapes and wines (*29*), epigallocatechin-3-gallate in green tea (*27*), ellagic acid, quercetin and resveratrol present in muscadine grapes (*30*), curcumin in turmeric spice (*31, 32*) and the list is continuously expanding.

Mango

Mango (*Mangifera indica* L.) is one of the most important tropical fruits consumed worldwide which has been cultivated in Asia for several years with India

being the main producer (*33*), accounting for almost 54% of the total mango production (*34*). Nowadays, this fruit is commercially grown in almost 87 countries making mangoes rank fifth in total production among major fruit crops (*34*).

Since mango is a climacteric fruit, the period of ripening is characterized by the production of ethylene and increase in the rate of respiration (*34*). During ripening, natural and characteristic color, aroma and taste are developed (*7, 34*). Due to its diversity in size, color and flavor, mangos are processed at every stage of growth. Green or raw mango fruits are used for a great diversity of products such as pickles and chutneys whereas ripe mango fruits are processed into canned and frozen slices and mango puree and pulp for the production of several products such as juices and nectar, jam, ready to serve drinks, among others (*34*).

The nutritional composition of mango is dependent on several factors such as variety, climate and stage of maturity (*7*). The edible portion of mango fruit contains around 0.82 mg protein, 1.6 mg fiber and 36.4 mg of vitamin C (ascorbic acid) and 1082 IU of vitamin A per 100 g of pulp, among other nutrients shown in Table 1.

Table 1. Nutritional composition of mango (*7, 34*)

Nutrient	Value per 100g
Proximates	
Water	83.46 g
Energy	60 kcal
Protein	0.82 g
Total lipid (fat)	0.38 g
Carbohydrate, by difference	14.98 g
Fiber, total dietary	1.6 g
Sugars, total	13.66 g
Minerals	
Calcium, Ca	11 mg
Iron, Fe	0.16 mg
Magnesium, Mg	10 mg
Phosphorus, P	14 mg
Potassium, K	168 mg
Sodium, Na	1 mg
Zinc, Zn	0.09 mg
Vitamins	
Vitamin C, total ascorbic acid	36.4 mg
Thiamin	0.028 mg
Riboflavin	0.038 mg
Niacin	0.669 mg
Vitamin B-6	0.119 mg
Folate, DFE	43 ug
Vitamin A, RAE	54 ug
Vitamin A, IU	1082 IU
Vitamin E (alpha-tocopherol)	0.9 mg
Vitamin K (phylloquinone)	4.2
Lipids	
Saturated fatty acids	0.092
Monounsaturated fatty acids	0.14
Polyunsaturated fatty acids	0.071

Mango varieties

Mango traditionally grows in warm and tropical climates. Fruits of the best quality are produced during dry seasons followed by rains; however some cultivars are capable to fruit in humid regions (*35*). The most important mango cultivars produced in different countries are shown in Table 2. Tharanathan et al. (*34*) indicates that Alphonso is the leading and best variety due to its excellent fruit quality characterized by a thin skin, soft flesh, low fiber content, sweet aroma and outstanding flavor besides its high content of vitamin C, β-carotene and minerals. The main mango cultivars commonly found in the United States are Ataulfo, Haden, Keitt, Kent and Tommy Aktins (*36, 37*). For research purposes, the variety Keitt will be described.

Mango variety Keitt

Mango Keitt is the most commercially important cultivar in the export mango industry of the western hemisphere after Tommy Aktins (*36, 38*). Knight et al. (*39*) describes the tree as medium size and vigorous whereas the fruit is generally greenish yellow with a pink or red blush. The skin is thick, tough and the flesh is firm and juicy, with some fiber, sweet and mild with a pleasant aroma (*39*). Campbell et al. (*38*) and Schnell et al. *(36)* indicates that this mango cultivar is resistant to diseases like anthracnose. Moreover, it is hardly affected by food processing steps like packing and shipping (*38*). Mango Keitt is shown in Figure 2.

Table 2. Mango cultivars and major producing countries (*35*)

Continent	Country	Cultivar
Africa	Cote d'lvoire	Amelie, Kent
	Egypt	Alphonso, Bullock's Heart, Hindi be Senara, Langra, Mabrouka, Pairie, Taimour, Zebda
	Kenya	Boubo, Ngowe, Batawi
	Malí	Amelie, Kent
	South Africa	Fascell, Haden, Keitt, Kent, Sensation, Tommy Atkins, Zill
Asia	Bangladesh	Aswina, Fazli, Gopal Bhog, Himsagar, Khirsapati, Langra
	China	Gui Fe, Tainong N° 1, Keitt, Sensation, Zill, Zhua, Jin Huang
	India	Alphonso, Banganapalli, Bombay, Bombay Green, Chausa, Dashehari, Fazli, Fernandian, Himsagar, Kesar, Kishen Bhog, Langra, Mallika, Mankurad, Mulgoa, Neelum, Pairi, Samar Behisht, Suvarnarekha, Totapuri, Vanraj, Zardalu.
	Indonesia	Arumanis, Dodol, Gedong, Golek, Madu, Manalagi
	Israel	Haden, Tommy Atkins, Keitt, Maya, Nimrod, Kent, Palmer
	Malaysia	Apple Rumani, Arumanis, Golek, Kuala Selangor 2, Malgoa
	Myanmar	Aug Din, Ma Chit Su, Sein Ta Lone, Shwe Hin Tha
	Pakistan	Anwar Ratol, Began Pali, Chausa, Dashehan, Gulab Khas, Langra, Siroli, Sindhri, Swarnarekha, Zafran
	The Philippines	Carabao, Manila Super, Tico
	Taiwan	Irwin, Jin-hwung, Keitt, Tommy Atkins, Tainong No.1, Tsar-swain
	Thailand	Nam Doc Mai, Ngar Charn, Ok Rong, Keow Savoey, Pimsen Mum

Table 2 Continued

Continent	Country	Cultivar
Australia	Australia	Calipso, Kensington Pride
North and Central America	Costa Rica	Haden, Irwin, Keitt, Mora, Tommy Atkins
	Dominican Republic	Haden, Keitt, Kent, Tommy Atkins
	Guatemala	Haden, Keitt, Kent, Tommy Atkins
	Haití	Francine, Madame Francis
	Mexico	Ataulfo, Haden, Keitt, Kent, Manila, Palmer, Sensation, Tommy Atkins, Van Dyke
	USA	Keitt, Kent, Tommy Atkins
South America	Brazil	Bourbon, Coite, Coquinho, Coracao, Espada, Haden, Itamaraca, Keitt, Mamao, Palmer, Rosa, Tommy Aktins, Uba, Van Dyke, Tommy Atkins, Uba, Van Dyke
	Colombia	Vallenato
	Ecuador	Haden, Keitt, Kent, Tommy Atkins
	Peru	Haden, Keitt, Kent, Tommy Atkins
	Venezuela	Haden, Keitt, Kent, Tommy Atkins

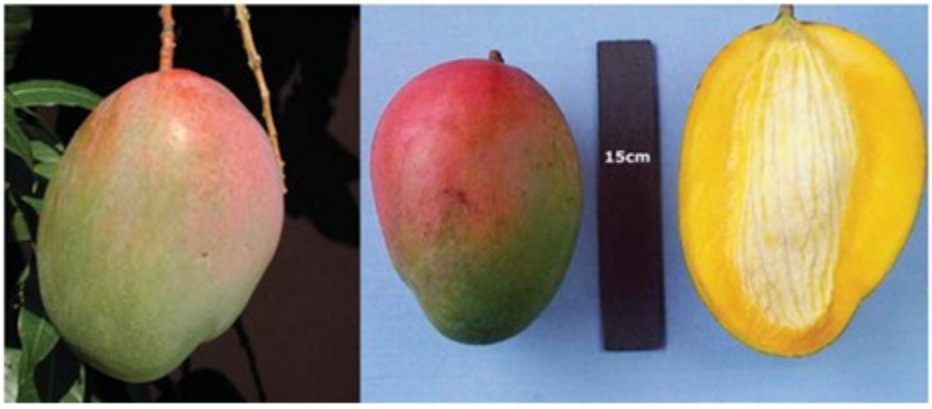

Figure 2. Keitt mangoes (*35*)

Major mango polyphenols

Among the polyphenols identified and characterized in the edible part of mango are flavonoids such as quercetin and kaempferol glycosides; and phenolic acids, galloyl glycosides and mangiferin (*6, 40*). These polyphenolic compounds are distributed among the different parts of the fruit including the pulp, seed kernel, peel, leaves and bark in which the concentration of these compounds varies greatly according to several factors such as variety, environmental factors, harvest conditions, processing and so on (*6*). The main polyphenols present in mangoes will now be described.

Gallic acid, ellagic acid and hydrolizable tannins

Gallic acid, ellagic acid and their derivatives are among the phenolic acids identified in mangoes, being gallic acid the major phenolic acid identified in mangoes followed by hydrolyzable tannins (*6, 40, 41*). Gallic acid is a trihydroxibenzoic acid which is found in both free form and as part of hydrolyzable tannins. This phenolic acid is distributed among the different parts of the mango fruit, for instance Scheiber et al. (*37*) found that the concentration of gallic acid in a commercial mango puree concentrate was 6.9 mg/kg. Soong and Barlow (*42*) reported that the amount of gallic acid in a mango seed extract ranged from 23 to 838 mg/100 g whereas Selles et al. (*43*) reported 226.2 mg/100 g of gallic acid in an extract from mango stem bark. The concentration of gallic acid is dependent of the mango variety, parts of the fruits from which it is extracted, degree of ripening and methods of extraction. Ellagic acid is a phenolic acid found in a great variety of fruits and vegetables. This compound, which is the dimeric

derivative of gallic acid, is found either in a free form or bound as ellagitannins (ellagic acid bound to a sugar molecule) (*6*). The concentration of this phenolic compound is also dependent on the mango variety, methods of extraction, among others. The chemical structure of gallic acid and ellagic acid are shown in Figure 3.

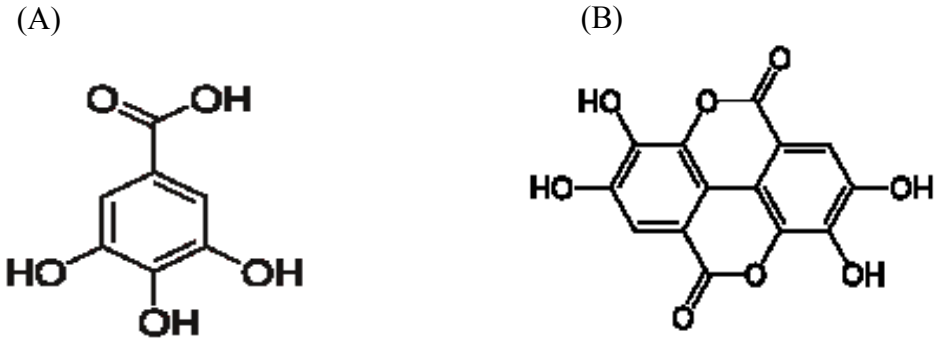

Figure 3. Chemical structure of (A) gallic acid and (B) ellagic acid

Besides gallic acid and ellagic acid, methyl and propyl gallate (Figure 4) have been reported to be found in mango (*6*). Selles et al (*43*) reported that a mango stem bark extract had about 445.2 mg/ 100g and 476.2 mg/100g of methyl gallate and propyl gallate respectively. Several *in vitro* and *in vivo* studies have indicated the antioxidant, anti-inflammatory, anti-microbial, anti-cancer and anti-mutagenic effects of gallic acid, ellagic acid, methyl and propyl gallate either when tested as isolated compounds or as part of a mixture. However, the use of pure or isolated compounds have been reported to be less effective than the use or application of a crude mixture due to the synergistic effect the compounds exert when they are combined.

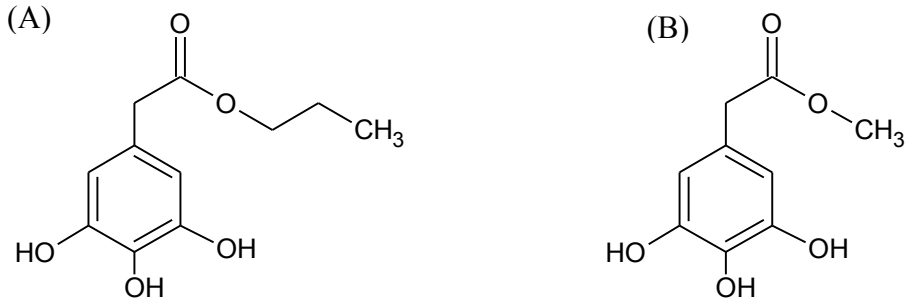

Figure 4. Chemical structure of (A) propyl gallate and (B) methyl gallate

Hydrolysable tannins present in mango are generally gallotannins (Figure 5) which are compounds formed when gallic acid esterifies and binds a polyol core such as glucose. Gallotannins are also considered as major antioxidant polyphenols found in mango (*41*). The presence of gallotannins varies in the different parts of mango and is dependent, among other factors, on fruit ripening; for instance Scheiber et al. (*37*) indicates that the concentration of gallotannins is higher in unripe fruits and seed. The gallotannins identified in the different parts of mango have different degree of galloylation ranging from mono to deca galloyl-O-glucosides. Noratto et al. (*40*) reported galloyl gluscosides in mango Haden ranging from 1 (mono-O-galloylglucosides) to 9 (nona-O-galloylglucosides) units whereas Ataulfo polyphenols had higher molecular weight gallotannnins ranging from 5 (penta-O-galloylglucosides) to 11 (undeca-galloylglucosides). Barreto et al. (*44*) reported tetra, penta and hexa-O-galloylglucosides in leaves, peels, kernels and barks of different cultivars of mango. Like gallic acid and ellagic acid, gallotannins present in mango are found to exert better anti-inflammatory, anti-carcinogenic and anti-microbial activity when tested as part of a crude mixture rather than used as isolated compounds; however, gallotannins alone

extracted from mango kernels exerted antibacterial activity in pathogenic microorganisms (*45*).

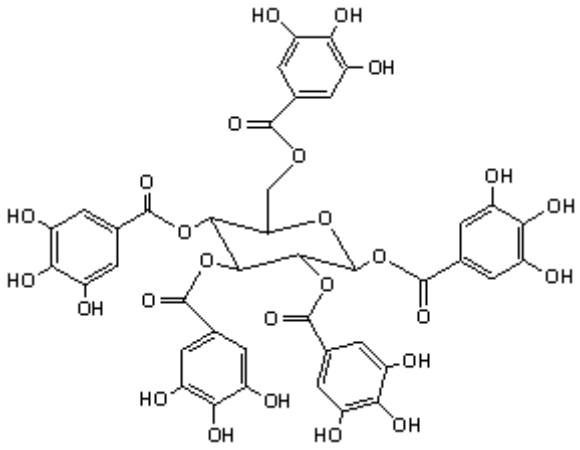

Figure 5. Chemical structure of gallotannins

Flavonoids

Flavonoids are compounds ubiquitous to plants and the most abundant polyphenols of our diet (*6, 46*). Flavonoids identified in mango include catechins, quercetin and kaempferol (*6, 40, 44*) which are shown in Figure 6. These compounds have shown strong antioxidant and scavenging properties conferring them medicinal properties. For instance, Selles et al. (*43*) identified a mango stem bark extract, which is used for the production of a Cuban nutritional supplement named Vimang, with high concentrations of catechins and epicatechins suggesting that they are in part responsible for the antioxidant activity of Vimang. Quercetin and its related glycosides have also been reported in mango pulp, leaves and peel (*6, 37, 44*).

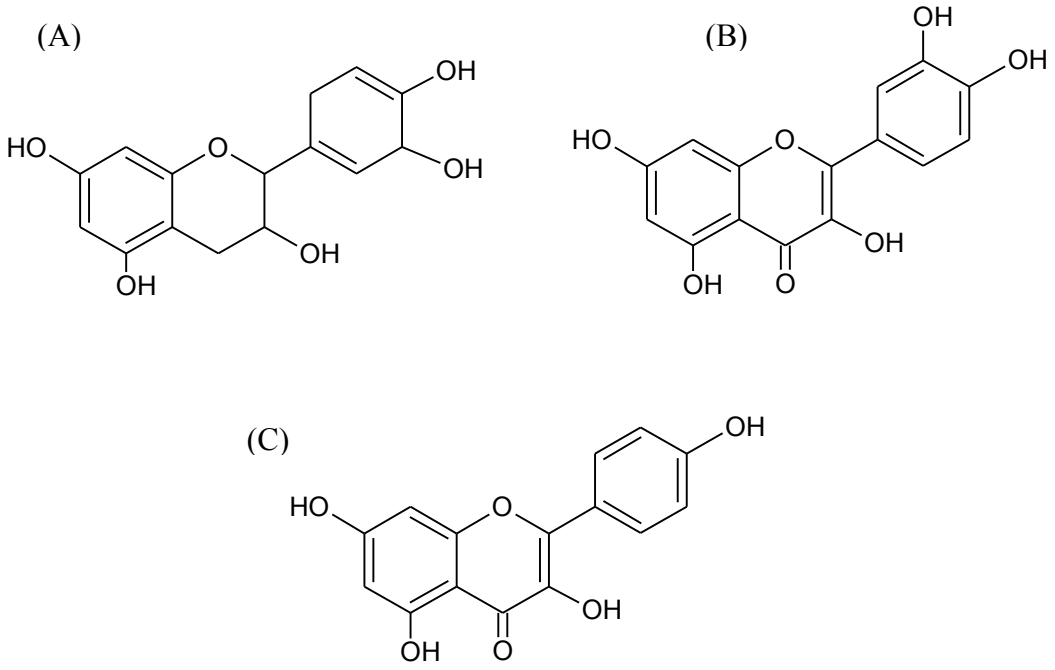

Figure 6. Chemical structure of (A) catechins, (B) quercetin and (C) kaempferol

Functional properties of mango polyphenols

Consumer interest in mango has been increasing in recent years due to the potential it may have towards the prevention of degenerative diseases, including cancer (*6*). Experimental data has shown that bioactive compounds present in mangoes exert anti-inflammatory, anti-carcinogenic, antiviral and antibacterial properties (*6, 44*) due to presence of polyphenolic compounds such as phenolic acids and flavonoids; and carotenoids which are considered to be the main group of bioactive compounds responsible for the health benefits described above. According to several authors, polyphenolic compounds found in mango have antioxidant properties that contribute to decrease oxidative stress which can lead to the onset of age related illnesses such as cancer (*6, 40, 44*).

The use of mango extracts in traditional medicine is widespread, for example Vimang, a mango stem bark extract has been used in Cuba for at least 10 years in the treatment of several diseases including cancer (*7, 34*). Furthermore, studies *in vitro* and *in vivo* conducted with individual polyphenols, such as gallic acid and penta galloylglucosides, present in mangoes have been shown antitumor activities including induction of apoptosis, inhibition of tumor growth and angiogenesis (*6, 47, 48*).

Noratto et al. (*40*) reported that Ataulfo and Haden mango polyphenols exerted anti-carcinogenic activity by inhibiting the growth of SW-480 colon cancer cells suggesting their use as chemopreventive agents. Wilkinson et al. (*49*) evaluated the bioactivity of mango flesh and peel extracts on MCF-7 breast cancer cells and found that the extracts were effective in inhibiting their proliferation. Percival et al. (*50*) concluded that polyphenols present in mango juice and methanol extracts of mango juice have antioxidant, growth-arresting and anti-promotion activity. Other studies have indicated that polyphenols from mango peel, flesh, seed, stem bark and leaves may play an important role in preventing the deleterious effects of free radicals which are, in part, responsible for the development of chronic diseases.

For the reasons described above, mango is not only a common tropical fruit but it could also be a potential and cheap source of dietary polyphenols with high antioxidative properties that could be used to reduce and/or prevent the incidence of chronic and degenerative diseases.

Breast Cancer

Breast cancer is one of the main causes of cancer death among women worldwide. The rates of this disease are high and expected to increase in the next decade (*1*). In the US, The American Cancer Society has estimated about 230,000 new cases of breast cancer and almost 40,000 deaths among women for 2013. According to the American Cancer Society, breast cancer is a disease that starts when cells begin proliferating without control and invading other tissues.

Types of breast cancer

Breast cancer can be classified, among other ways, as non-invasive and invasive breast cancer. Ductal and lobular carcinoma are the most common types of invasive breast cancer which are responsible for 75% and 15% of all cases in the US respectively (*2*). Breast tumors can be a mixture of invasive and non-invasive (in situ) carcinomas which are described as follows:

- ***Ductal carcinoma in situ (DCIS)***: The American Cancer Society indicates that ductal carcinoma initiates in the tubes carrying breast milk to the nipple (ducts of the breast) and have not spread to other tissues. At this stage women can be treated and cured but if it is untreated, it may progress into invasive breast cancer (*51, 52*).
- ***Invasive ductal carcinoma (IDC)***: Also known as infiltrating ductal carcinoma, it is considered the most common type of breast cancer. At this stage, cancer cells start invading other tissues of the breast from where it starts invading or spreading

to other parts of the body (metastasis) through the bloodstream or lymphatic system (*52*).

- ***Invasive (infiltrating) lobular carcinoma (ILC):*** This cancer starts in the lobules or glands, which is the place where milk is produced. Cancer cells start spreading through the wall of the lobules and invading other parts of the body causing metastasis (*52*).
- ***Inflammatory breast cancer (IBC):*** This cancer is considered a rare and aggressive form of invasive breast cancer. The American Cancer Society indicates that the symptoms of IBC are caused when cancer cells block the lymph vessels causing redness, swelling and tenderness of the breast skin. IBC is harder to diagnose compared to other breast cancers because a breast lump is rarely formed making it difficult to visualize in a mammogram (*52*).
- ***Estrogen positive and estrogen negative breast cancer:*** Estrogen is a hormone that plays an important role in breast cancer development and progression. Since most breast cancer tumors grow in response to estrogen, they are known as estrogen receptor positive cancer or ER (+). According to the American Cancer Society almost 75% of breast cancers are ER+. In contrast, when breast cancer tumors do not grow in response to estrogen, they are estrogen receptor negative or ER (-). HER2 (Human epidermal growth factor receptor 2) is another common type of breast cancer in women accounting for around 20-25% of breast cancer cases in the US. HER2 is a gene involved in the growth and division of cells responsible for the aggressiveness and fast growth of breast cancer tumors; therefore it has become a

significant biomarker and target in the treatment and therapy of breast cancer (*51, 52*).

Risk factors

The risk factors associated with this disease can be classified into modifiable (diet, drinking and physical activity) and non-modifiable (age, genetic factors and family history) factors.

Modifiable risk factors

Accumulative research has shown that modifiable risk factors play an important role in the prevention of all types of cancer, including breast cancer. Among these risk factors, diet is considered to have a preventive and protective role against several types of cancer. It has been estimated that 30-40% of cancer diseases can be prevented with a healthy lifestyle and diet (*53*). Western type diet is characterized by a high intake of fat and refined foods and frequently correlated with an increased incidence of obesity, cardiovascular diseases and cancer (*54, 55*). For example, Wu et al. (*56*) conducted a case-control study of Asian women who migrated to the US and US-born Asian-American women and concluded that the breast cancer rates between these groups depended not only on menstrual or reproductive factors but also the physical and diet differences had a major impact. It has also been reported that an elevated body mass index (BMI) and obesity are major risk factors for breast cancer development and progression (*57*). Alcohol consumption has also been associated with breast cancer; for

example a moderate alcohol intake could increase breast cancer risk by around 7% (*57, 58*). Physical activity might affect hormonal concentration and energy balance that is why is positively correlated with a decrease in breast cancer incidence (*59*). A prospective observational study evaluated the effects of physical activity among women with breast cancer and concluded that it could reduce the risk of death among women diagnosed with breast cancer compared with sedentary women (*60*).

Non modifiable risk factors

The incidence of breast cancer increases with age; however it decreases in women over 50 years old. (*58*). It has also been reported that women who experience an early period (before 12) and a late menopause (typically after 55) are prone to develop breast cancer (*58*). The American Cancer Society indicates that the chances of getting breast cancer are higher among women whose close relatives (mother and/or sister) have had this disease previously. Weber et al. (*61*) evaluated the influence of family history and breast cancer risk and found a two fold increase in breast cancer risk among women whose mother or sister was diagnosed with the same disease. Breast cancer is also thought to be linked to genetic factors, specially inherited changes or mutations in some genes (*58*). BRCA1 and BRCA2 are genes which are expressed in breast cells and among other functions, prevent DNA damage. It has been reported that changes in these genes can be responsible for an 80% chance of breast cancer in women (*58*).

Polyphenols and breast cancer

Several dietary polyphenols have been tested in vitro and in vivo in order to determine their potential role in growth inhibition, proliferation and apoptosis in breast cancer cells. It has been reported that polyphenols from wine (*62*), pomegranate (*63, 64*) and green tea (*65, 66*) exerted cytotoxic, antiproliferative and anti-tumor properties on breast cancer cell growth suggesting a potential use of dietary polyphenols in the prevention of breast cancer. However, their mechanism of action as anti-tumor agents needs to be further elucidated for clinical applications in patients.

The anticarcinogenic effects of resveratrol is well documented, in fact several *in vitro* and *in vivo* studies have addressed the effects of resveratrol in several types of cancer, including breast cancer (*67*). Resveratrol has reported to be efficient in both hormone sensitive and hormone resistant cancer cells by exerting anti-inflammatory, anti-proliferative, and pro-apoptotic effects (*62, 67*).

The cytotoxicity of pomegranate polyphenols has been evaluated in several types of cancer indicating that its relevant compounds have anti-inflammatory and anti-carcinogenic properties *in vitro* and *in vivo.* Banerjee et al. (*63*) evaluated the effects of pomegranate polyphenols in BT-474 and MDA-MB231 breast cancer cells and nude mice bearing BT-474 as xenografts and concluded that polyphenols compounds found in pomegranate showed cytotoxic and anti-inflammatory activities mainly by regulating the miR-27a-ZBTB10 and miR-155-SHIP-1 pathways.

Green tea polyphenols and its component epigallocatechin gallate were tested using both breast cancer *in vitro* and *in vivo* models. Treatment with green tea

polyphenols inhibited proliferation and induced apoptosis in MDA-MB231 breast cancer cell *in vitro* and *in vivo* since the expression of cyclin D, cyclin E, CDK 4, CDK 1 and PCNA, which are genes involved in cell cycle progression, were downregulated (*65*). Likewise, Kavanagh et al. (*66*) evaluated the anticarcinogenic effects of green tea polyphenols *in vitro* and *in vivo* and reported that these polyphenols inhibited HS578T and MDA-MB231 breast cancer cells proliferation and exerted chemopreventive effects on carcinogen-induced mammary carcinogenesis in female Sprague-Dawley rats.

Inflammation and Cancer

Inflammation is a natural response from the body to irritation, injury or infection generally characterized by pain, redness and swelling among other symptoms (*68*). There are two stages of inflammation: acute and chronic inflammation. Acute inflammation is an initial step of inflammation regulated through the activation of the immune system and it generally lasts for a short period of time (*69*) whereas chronic inflammation can foster a wide variety of diseases such as cancer, diabetes, cardiovascular and neurological disorders (*68, 70*). Chronic inflammation is triggered by multiple factors including bacterial, viral and parasitic infection, moreover it has been suggested that environmental and lifestyle factors can also play a pivotal role in chronic inflammation (*68, 71*). The longer the inflammation lasts, the higher the risk of carcinogenesis (*71*). Chronic inflammation has been linked to several steps involved in carcinogenesis since most cancers arise from sites of infection, chronic irritation and

inflammation. Moreover, inflammatory cells have the ability to orchestrate a tumor microenvironment and to induce tumor development as well (*68-70, 72*).

Several pro-inflammatory cytokines, chemokines and inflammatory enzymes are associated with chronic inflammation. These genes play an important role in cellular and biological processes such as apoptosis, proliferation, angiogenesis, invasion and metastasis (*68*). The genes involved in these processes are several and include TNF-α (tumor necrosis alpha factor) and members of its family such as interlukines (IL-1β, IL-6, IL-8, IL-18) and chemokines (MMP-9, VEGF, COX-2) all of them regulated by the transcription factor NF-κB which is considered a master regulation of inflammation and active in most tumors (*3, 68, 70-72*). Given this circumstances, it is suggested that those compounds able to suppress the expression of NF-κB or its downstream genes might have a potential in the prevention of cancer.

PI3K/AKT/mTOR Pathway

The signaling pathway including phosphatidylinositol 3-kinase (PI3K), protein SER/Thr-kinase (AKT) and mammalian target of rapamycin (mTOR) has been described as a key regulator in several cellular and biological processes such as cell growth, proliferation and survival (*73*). Moreover, is considered as a potential therapeutic target in several types of cancer, including breast cancer since its aberrant expression controls and regulates several mechanisms such as breast cancer cell proliferation and anti-cancer drug resistance (*74-77*).

The activation of the PI3K/AKT/mTOR pathway (Figure 7) is triggered by tyrosine kinase growth receptors including receptor of the ErbB family, fibroblast growth receptor (FGFR) or insulin-like growth factor 1 receptor (IGF-1R) as well as by genetic mutations and amplification of key pathway component (*73, 77*). Once PI3K is activated, it starts a signal transduction cascade that promotes cancer cell growth, survival and metabolism which contributes to tumor progression (*73*).

Akt, a serine–threonine kinase, is aberrant activated in response to PI3K. Ghayad et al. (*77*) indicates that when AKT is entirely activated it translocates to the nucleus to phosphorylate its downstream substrates involved in cell growth and survival, apoptosis and translation. The downstream substrate of AKT is mTOR, a serine-threonine protein kinase that controls and regulates cell growth and proliferation (*78*). mTOR exists in two protein complexes within the cell, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (*75, 77, 78*). mTORC1 is activated and regulated by a number of signaling pathways, including by growth factor signaling through PI3K activation of AKT (*75, 78*). mTORC1 induces protein translation by phosphorylating its downstream effectors p70S6K (ribosomal protein S6 kinase) and 4E-BP1 (eIF4E-binding proteins) (*77, 78*).

Evidence suggest that mTORC1 regulation is important from a therapeutic point of view since some PI3K inhibitors block both PI3K and mTOR while others only block PI3K, therefore those substances or compounds that have dual inhibitor properties might have an advantage in cancer therapy and treatment (*73*).

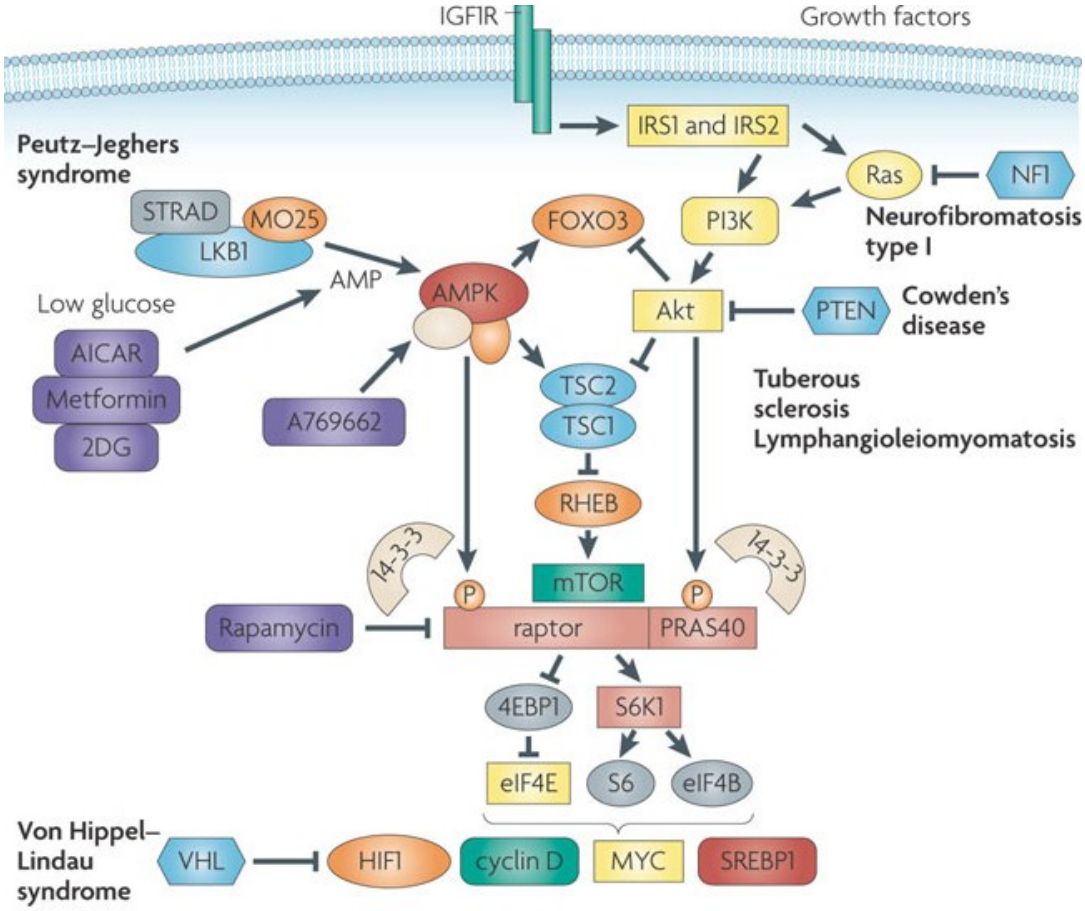

Figure 7. PI3K/AKT/mTOR signalling pathway (*79*)

The activation of the PI3K/AKT/mTOR pathway is also related to the inhibition of the tumor suppressive activity of PTEN (phosphatase tensin homolog), which loss of function or decreased expression is associated with several types of cancers including breast carcinoma. Carracedo et al. (*80*) states that PTEN is a main component in the regulation of PI3K signaling, therefore perturbations or alterations in its level or function can result in dramatic impacts on this signaling pathway.

The PI3K/AKT/mTOR pathway has been implicated in the regulation of the hypoxia inducible factor 1 alpha (HIF-1α), the master control transcription factor of the cellular response to hypoxia (*81, 82*). The overexpression of HIF-1α has been demonstrated in many cancers, including breast cancer (*81*). The activity of HIF-1α in cancer cells and tumors depends on the availability of HIF-1α subunit, which levels increase under hypoxia conditions (inadequate oxygen supply) (*83*); however, under normoxic conditions (regular oxygen supply), HIF-1α can be up-regulated by growth factors like insulin growth factor (IGF) and cytokines such as TNF-α (*81*). In addition, HIF-1α activation also occurs through the stimulation of oncogenes and/or inactivation of tumor suppressor genes such as PTEN (*81-83*). The overexpression of HIF-1α has been linked to angiogenesis since HIF-1α is a major upstream regulator of VEGF (major protein involved in angiogenesis); consequently suppression of HIF-1α/VEGF is becoming a potential therapeutic target for cancer therapy (*81-84*).

Role of polyphenols in PI3K/AKT/mTOR pathway

Since the PI3K/AKT/mTOR pathway is negatively stimulated in many cancers, its inactivation constitutes an attractive target for the development of anti-cancer compounds (*77*) which comprises several therapeutics such as dual PI3K-mTOR inhibitors, PI3K inhibitors, AKT inhibitors and mTOR inhibitors (*73*).

Several polyphenols have been shown to inhibit the activation of the PI3K pathway through the down-regulation of AKT and mTOR, for instance Van Aller et al. (*85*) concluded that epigallocatechin gallate in green tea has anti-cancer properties by

targeting the PI3K/AKT/mTOR pathway in MDA-MB231 breast cancer cells. Another study indicated that cocoa polyphenols repressed the TNF-α induced phosphorylation of Akt and blocked the activation of its downstream kinases such as p70S6K ribosomal protein in JB6 mouse epidermal cells. Moreover, cocoa polyphenols decreased TNF-α-induced up-regulation of VEGF by inhibiting PI3K, suggesting its chemopreventive potential (*86*). Resveratrol was also shown to be a potential inhibitor of the PI3K/AKT/mTOR signaling pathway in human U251 glioma cells (*87*). Another study suggested that potential use of curcumin in colorectal cancer radiation therapy since it inhibited NF-Kb and attenuated the effect of irradiation-induced signaling through the PI3K/AKT/mTOR and NF-Kb pathway in human intestinal microvascular endothelial cells (*88*).

Micro-RNAs and Polyphenols

Small non-coding RNAs (20-24 nucleotides in length) or microRNAs (miRNAs) modulate gene expression at the post-transcriptional level in a sequence-specific manner (*89, 90*). Since miRNAs regulate the expression of several genes in normal cells, they are involved in important cellular and biological processes such as differentiation, cell growth/proliferation and cell death (*89*). miRNAs have tumor suppressor and oncogenic functions (*89*), therefore they are considered as potential targets in human diseases, including cancer.

Micro-RNA 126 (miRNA-126) is expressed in normal human breast tissue (*91*) and its expression is generally lost in several types of cancer, in fact miRNA-126 is one

of the miRNAs underexpressed in breast cancer cells (*92*) which has been correlated with a very poor metastasis free-survival in breast cancer patients (*91*). The gene expression of miRNA-126 has been attributed to different roles including inflammation and carcinogenesis (*93*). One of the potential targets associated with miRNA-126 is the regulatory subunit p85 beta (p85β) involved in the PI3K signaling pathway. A study conducted in patients with lung cancer reported an association with the overexpression of miRNA-126 and a decrease expression of phosphorylated AKT suggesting a regulation of the PI3K/AKT pathway by miRNA-126 (*94*).

Among the miRNAs identified in a wide range of cancers, micro-RNA 21 (miRNA-21) plays an important role in carcinogenesis, for instance it has been reported that miRNA-21 is up-regulated in a wide range of cancers, especially breast cancer (*90, 95*). Therefore, knockdown of miRNA-21 has been linked to cell growth inhibition and increased apoptosis in vitro and in vivo (*90, 95*). In addition, miRNA-21 is one of the most anomaly and commonly increased miRNAs in cancer, and plays essential roles in cancer growth, proliferation, migration, and metastasis by targeting a wide variety of genes such as PTEN (phosphatase tensin homolog) which is considered as an important downstream target of miRNA-21 (*96*). The phosphatase tensin homolog is a tumor suppressor which expression is lost in various cancers (*80*). Inactivation of PTEN leads to an increase expression of PI3K signaling and Akt phosphorylation, so it has proposed that PTEN is a major player in the regulation of PI3K signaling, thus alterations in its levels or function might have considerable impact on this pathway (*80, 96*).

Recent studies have shown that dietary factors influence the expression of miRNAs. Moreover, plant polyphenols have been investigated in order to determine their interaction and potential modulation of particular miRNAs in several *in vitro* and *in vivo* models (*63, 97, 98*) suggesting that their up or down regulation can reduce the risk of certain chronic disease.

CHAPTER III

ANTI-INFLAMMATORY EFFECTS OF MANGO KEITT POLYPHENOLS IN MCF-12A NON-CANCER BREAST CELLS: POTENTIAL INVOLVEMENT OF miRNA-126

Summary

Mango is a tropical fruit characterized for its unique aroma and flavor. In addition of its olfactory properties, mango may be considered as a functional fruit since its regular consumption is associated with potential health benefits. In fact, several studies have reported that mango is a rich source of polyphenols which may be used as antioxidant and anti-inflammatory agents in the prevention of chronic diseases such as cancer. Several studies have reported the anti-inflammatory and anti-cancer effects of mango polyphenols *in vitro* and *in vivo*; however, their capacity to regulate microRNAs has yet.to be elucidated. The objective of this study was to investigate the anti-inflammatory activity of mango Keitt polyphenols in MCF-12A non cancer breast cells through the modulation of the PI3K/AKT/mTOR inflammatory pathway and to evaluate the involvement of miRNA-126 as a potential underlying mechanism. Mango Keitt polyphenol extract (ME) did not show inhibitory growth effects within a concentration range of 2.5-10 μg GAE/ml in MCF-12A non-cancer breast cells. In addition, ME counteracted the production of reactive oxygen species (ROS); however, no significant differences were observed between different concentrations of ME. A significant decrease in the gene expression of NF-κB and related inflammatory downstream genes

was observed when MCF-12A non-cancer breast cells were challenged with TNF-α (10 ng/ml). ME also attenuated the gene and protein expression of PI3K, AKT and mTOR and these results were accompanied by an up-regulation of miRNA-126 within a concentration range of .2.5-10 μg GAE/ml. These results were confirmed by transfecting MCF-12A non-cancer breast cells with a specific antagomir for miRNA-126 in which ME treatment reverted the effects of the antagomir. Moreover, ME down-regulated the gene expression of PI3K (p85β) in miRNA-126 knockout cells as well. Overall, polyphenols present in mango Keitt may have the capacity to combat inflammatory challenges in MCF-12A non-cancer breast cells by regulating the genes involved in the PI3K/AKT pathway such as NF-κB and mTOR. In addition, mango Keitt polyphenols are likely to play an important role in the regulation of miRNA-126, which effects are mediated by the PI3K/AKT pathway.

Introduction

The consumption of fruits and vegetables is positively associated not only with a healthy and balanced diet but also with the prevention of different diseases such as cardiovascular and neurodegenerative disorders and some types of cancer. In addition, a diet rich in fruit and vegetables have a significant impact in the prevention of being overweight or obese. Bioactive compounds from several fruits, vegetables, beverages and spices are considered to be the major factors implicated in the prevention of such chronic diseases.

Mango (*Mangifera indica* Linn.) is a tropical fruit that has received the denomination of "*the king of fruits"* because of its intense aroma, tasty flavor and high nutritional value, since it is a good source of vitamin A and C, fiber and minerals (*34*). Moreover, mango is considered to be a rich source of polyphenols which have shown strong antioxidant activity capable of counteract the deleterious effects of free radicals which lead to the onset of degenerative diseases, in particular cancer. Experimental data has shown that bioactive compounds present in mangoes may exert anti-inflammatory, anti-carcinogenic, antiviral and antibacterial properties due to the presence of phenolic acids, flavonoids, hydrolysable tannins (gallotannins), carotenoids and mangiferin which are considered to be the main group of polyphenols identified in the edible part of mango (*6, 7, 44*). In fact, several studies have reported the anticancer activities of mango polyphenols in colon, breast and cervical cancer cells (*40, 49, 99*).

Chronic inflammation has been linked to several steps involved in carcinogenesis since most cancers arise from sites of infection, chronic irritation and inflammation. Increasing evidence indicates that estrogen exposure is associated with an increased risk of developing breast cancer. Additionally, other hormones like insulin-growth factors (IGFs) have a substantial effect on breast cancer growth. Previous studies have shown that IGF-I is a potent mitogen and may act synergistically with estrogen to stimulate cell proliferation and activate signaling pathways (*100*).

During inflammation in the breast, cells have the capacity to orchestrate a tumor microenvironment leading to several risk factors associated with breast cancer as well as the development of aggressive breast tumors (*4*). Several pro-inflammatory cytokines,

chemokines and inflammatory enzymes are activated as a result of chronic inflammation. Several genes are involved in this process and include TNF-α (tumor necrosis alpha factor) and members of its family such as interlukines (IL-1β, IL-6, IL-8, IL-18). All of them listed are regulated by the transcription factor NF-κB which is considered the master regulator of inflammation (*3, 68-70, 72*). Previous studies reported the modulation of inflammatory genes by polyphenols from different sources suggesting their potential role as anti-inflammatory agents (*98, 101, 102*).

The PI3K/AKT signalling pathway has been described as a key regulator in several cellular and biological processes (*73*), however, its role during inflammation in non-cancer breast cells has not been reported extensively. During carcinogenesis the PI3K/AKT cascade has a major effect in cell survival pathways through phosphorylation of dowstream targets such as NF-κB and mamalian target of rapamycin (mTOR) (*103*). Polyphenols may be able to supress the PI3K/AKT pathway and related dowstream genes in an inflammatory microenvironment. For instance, cocoa polyphenols repressed the TNF-α induced phosphorylation of Akt and blocked the activation of its downstream kinases such as p70S6K ribosomal protein in JB6 mouse epidermal cells (*86*).

The identification of miRNAS and their specific targets have emerged as novel players in anti-inflammatory therapeutic strategies, but the role of polyphenols in the regulation of miRNAs has not been extensively investigated. Micro-RNA 126 (miRNA-126) is involved in different physiological and pathological processes, including inflammation and cancer (*93*). One of the potential targets associated with miRNA-126

is PI3K; indeed experimental data has shown the potential role of tumor suppressor miRNA-126 in inflammation processes by targeting PI3K.

The main objective of this study was to investigate the anti-inflammatory activity of mango Keitt polyphenols in MCF-12A non cancer breast cells through the modulation of the PI3K/AKT signaling pathway and related downstream genes such as NF-κB and mTOR and to evaluate the involvement of miRNA-126 as a potential underlying mechanism.

Materials and Methods

Plant material

Mango (*Mangifera indica* L.), variety Keitt, was provided by the National Mango Board (U.S). This fruit was obtained at a green stage and was allowed to ripen at room temperature until a moderate ripe stage was reached. Fruit was manually peeled to remove skin and seed and only the pulp was used for polyphenol extraction. Pulp was diced, vacuum sealed and stored at -20 C until needed.

Cell line

MCF-12A breast epithelial cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended by ATCC. MCF-12A breast cells were cultured using a 1:1 mixture Dulbecco's modified Eagle's medium and Ham's F12 medium, 20 ng/ml Human epidermal growth factor, 100 ng/ml

cholera toxin, 0.01 mg/ml bovine insulin, 500 ng/ml hydrocortisone, 95% and horse serum, 5%. Cells were maintained at 37° C with a humidified 5% CO_2 atmosphere.

Chemicals, antibodies and reagents

Standards for HPLC-MS analysis were obtained from Sigma-Aldrich (St Louis, MI). The Folin-Ciocalteu reagent was purchased from MP biochemical, LLC (Solon, Ohio). 2'7'-dichlorofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO) and Tumor Necrosis Factor alpha (TNF-α) were purchased from Sigma (St Louis, MI). Bradford reagent was obtained from BioRad (Hercules, CA), antibodies against NF-kB p65, phospho-NF-kB p65 and β-actin were obtained from Cell Signaling Technology (Beverly, MA).

Mango polyphenols extraction

Mango pulp was extracted as previously described with slightly modifications (*40*). Briefly, mango pulp was thawed and homogenized in a blender and extracted in a ratio of 500g of pulp to 1.5 L of a solvent mixture (methanol:ethanol:acetone, 1:1:1) for 1 h. at room temperature. After 1 h. incubation with solvents, solids were centrifuged at 3000 rpm for 10 minutes followed by filtration and extracted twice following the same procedure. The solvents were removed under reduced pressure at 40°C. Mango polyphenols were partitioned using a $20cm^3$ Waters C18 cartridge (Waters Corporation, Milfords, MA) previously conditioned with methanol 100% and acidified (0.01% HCl) water. Those compounds that were not adsorbed in the cartridge were extracted with

ethyalcetate in a separatory funnel. The ethylacetate fraction and methanol phase from the C18 cartridges were combined and the solvents were removed under reduced pressure until dryness was reached. The dried extract was reconstituted in DMSO (0.2%) for cell culture assays. A control with 0.2% DMSO was included in all assays.

Separations were made on an Acclaim™ C18 column (Bannockburn, Il), (250 x 4.6 mm, 5 μm) at room temperature. The mobile phase consisted of 0.1% formic acid in water (Phase A) and 0.1% water (Phase B). A gradient program at 0.4 mL/min initially ran Phase B at 0%, for 3 minutes, 21% Phase B in 20 min, from 21 to 35% Phase B in 30 min, and 35 to 49% Phase B in 50 min, 49% to 70% Phase B in 70 minutes before returning to initial conditions. Detection was at 280 and 360 nm for benzoic and cinnamic acids/flavonoids, respectively. Compounds were tentatively identified based on mass spectrometric analysis. This was performed on a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer with an electrospray ionization probe in negative ion mode under the following conditions: sheath gas (N2), 40 units/min; auxiliary gas (N2), 5 units/min; spray voltage, 4.5 kV; capillary temperature, 300°C; capillary voltage, 7.0 V; tube lens offset, 40.0 V, source current at 80.0 μA.

Determination of total soluble phenolics

Total soluble phenolics were measured by the the Folin-Ciocalteu assay (*104*) at 726 nm. Gallic acid was used as a standard to quantify total soluble phenolics by linear regression. Results were expressed as mg of gallic acid equivalents (GAE)/ gr of pulp.

Antioxidant capacity

Antioxidant capacity was assessed by the oxygen radical absorbance capacity (ORAC) assay using a BGM Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission), as previously described (*105*). Results were expressed in μmol Trolox equivalents (TE)/ gr. of mango pulp.

Cell proliferation

Cells (1.5 x 10^4) were seeded onto a 24 well plate and incubated for 24 h to allow cell attachment. Cells were treated with different mango polyphenol concentrations (2.5 – 10 μg GAE/ml). Cell proliferation was determined after 48 h incubation with mango Keitt polyphenol extract using a cell counter (Z2 Series Beckman Coulter, Fullerton, CA). Cell counts were expressed as a percentage of control cells as previously described (*40*).

Generation of reactive oxygen species

Determination of reactive oxygen species was assessed as previously described (*105*) using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) assay with slightly modifications. MCF-12A cells (1.5 x 10^4) were seeded in a black bottom 96 well plate and treated with different mango polyphenol concentrations (2.5 - 10 μg/ml) for 24 h. After incubation cells were challenged with TNF-α (10ng/ml) for 3 h at 37 °C to induce ROS production which was detected using DCFH-DA (10 μM) for 30 min at 37 °C. Fluorescence signal was measured at 520 nm emission and 480 nm excitation in a

FLUOstar Omega microplate reader (BMG Labtech Inc, Durhan, NC). After monitoring the fluorescence signal, cell counting was determined as described before (*106*). Briefly, MCF-12A cells were washed twice with PBS and fixed with 100% methanol for 3 min. After removal of methanol, cells were stained with 1mg/ml Janus green for 3 min, washed twice with PBS and 50% methanol before determining the cell counting at 654 nm in a plate reader. Relative fluorescence units (RFU) were normalized to the cell counting measured by the absorbance.

Real time PCR analysis of mRNA and microRNAs

MCF-12A cells ($3x10^5$ cells/well) were seeded in 6-well plates and incubated for 24 h to allow cell attachment before treatment with different mango polyphenol concentrations (2.5 – 10 μg GAE/ml) for 24 h. mRNA and miRNA were isolated using the mirVana™ miRNA Isolation Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommended protocol. The quality and quantity of RNA samples were assessed using the NanoDrop ® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm. The isolated RNA was used to synthetize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) following the manufacturer's recommended protocol. Each primer tested was designed using Primer Express software (Applied Biosystems, Foster City, CA); homology searched by an NCBI BLAST and the specificity was examined by a dissociation curve analysis. Real time PCR reactions were conducted using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT Fast

Real Time PCR System (Applied Biosystems, Foster City, CA). Primers were purchased from Integrated DNA Technologies, Inc. (San Diego, CA) and the sequences of the primers used were as follows:

TBP F: 5'- TGCACAGGAGCCAAGAGTGAA-3'

TBP R: 5'- CACATCACAGCTCCCCACCA-3'

NFKB F: 5'- TGGGAATGGTGAGGTCACTCT-3'

NFKB R: 5'- TCCTGAACTCCAGCACTCTCTTC-3'

IL-1B F: 5'- CTTCAGGCAGGCCGCGTCAG-3'

IL-1B R: 5'- TGCTGTGAGTCCCGGAGCGT-3'

IL-6 F: 5' - AGGGCTCTTCGGCAAATGTA-3'

IL-6 R: 5'-GAAGGAATGCCCATTAACAACAA-3'

TNF-α F: 5'-TGTGTGGCTGCAGGAAGAAC-3'

TNF-α R: 5'-GCAATTGAAGCACTGGAAAAGG-3'

VCAM-1 F: 5'- ACAGAAGAAGTGGCCCTCCAT-3'

VCAM-1 R: 5'- TGGCATCCGTCAGGAAGTG-3'

PI3K F: 5'- CCTGGCACCTATGTGGAGTT-3'

PI3K R: 5'- ACATCAGGTGGGGAGAACTG-3'

AKT F: 5'-TCCCGAGGCCAAGTCCTT-3'

AKT R: 5'-CCGCCAAGCCTCTGCTT-3'

mTOR F: 5'- CAAACAGTTCACCCTCAGT-3'

mTOR R: 5'-GCTGCCACTCTCCAAGTTTC-3'

Quantification and analysis of miRNA-126 and RNU6B (endogenous control) were assessed using the Taqman® MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA) as previously described (*105*). Briefly, the reverse transcription samples were diluted in a 1:15 ratio and amplified with Taqman ® Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Foster City, CA).

Transfection assay

Cells ($3x10^5$) were seeded in 6-well plates and transfected with 20nM miRNA-126 (Dharmacon, Lafayette, CO) as previously described (*105*) using Lipofectamine 2000 (Invitrogen, Carlsband, CA) for 6 h. After transfection, cells were treated with 10 μg GAE/ml of mango Keitt polyphenol extract for 24 h. and collected for RNA extraction.

Protein expression

Cells were seeded ($3x10^5$ cells/well) in 6-well plates and incubated for 24 h. to allow cell attachment before treatment with different mango polyphenol concentrations (2.5 – 10 μg GAE/mL) for 24 h. Cells were harvested and cell lysates were obtained using the Pierce Ripa buffer (25mM Tris®HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) supplemented with Halt protease and phosphatase inhibitors (Thermo Scientific, Rockford, IL.). Protein content was assessed using the Bradford reagent (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Proteins were separated on a 10 and 20% SDS-PAGE at 120 V for 1 h. and transferred to

PVDF membrane (Bio-Rad, Hercules, CA) as previously performed (*105*). Membranes were blocked with 5% non- fat milk in 0.1% Tween-PBS (T-PBS) solution for 1 h. and incubated with primary antibodies (1:1000) in 3% bovine serum albumin in T-PBS overnight at 4C with gentle shaking. After 3 washing steps with T-PBS for 5 minutes each, membranes were incubated with secondary antibodies (1:2000) in 5% non-fat milk dissolved in T-PBS for 1 h. Membranes were washed three times for 15 minutes and proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY).

The Luminex® assay was assessed using an 11-plex AKT/mTOR phosphoprotein magnetic bead kit (Millipore, Billerica, MA) following the manufacturer's protocol. Data were analyzed using Luminex xPonent 3.0 software.

Statistical analysis

Data from *in vitro* experiments were analyzed by one-way-analysis of variance (ANOVA) using SPSS (SAS Institute Inc., Chicago, IL). Data represent mean values with their standard deviations (SD) or standard error (SE), corresponding to three or more replicates. Differences were deemed significant at $p < 0.05$ using a Duncan pairwise comparison. For transfections with antagomir miRNA-126, differences were deemed significant at $p < 0.05$ using a *t*-student comparison.

Results and Discussion

Chemical composition

Mango polyphenol extraction was performed several times and after several extractions, two different mango extracts (mango extract A and mango extract B) were obtained. The differences between them were mainly the method of extraction and the mango harvest used to conduct the polyphenol extraction. As far as the method of extraction, mango extract A was treated with pectinase to get a high yield of mango juice and facilitate the process of polyphenol extraction whereas extract B did not receive this treatment. As far as the harvest, the polyphenol extraction was conducted using two different mango harvests.

Several studies have reported that mango varieties are a rich source of a diverse class of polyphenols such as gallotanins, gallic acid, galloylglucosides, and flavonoids. In addition, mangiferin and a complex mixture of other polyphenols have also been reported. The representative chromatographic profile of mango extract A (Figure 8) confirmed the presence of phenolic acids (i.e. gallic acid), hydrolysable tannins and many other phenolic acids which were reported in previous studies (*40, 44*). Hydrolyzable tannins (gallotannins) were identified by HPLC-ESI-MS^n analysis. Polyphenols compounds with an elution pattern after 45 min included gallotannins ranging from penta-O-galloyglucose (939 Da.) to nona-O-galloyglucose (1547 Da.). In general, the polyphenolic composition of fruits is dependent on several factors such as variety, climate, harvesting, processing and storage conditions.

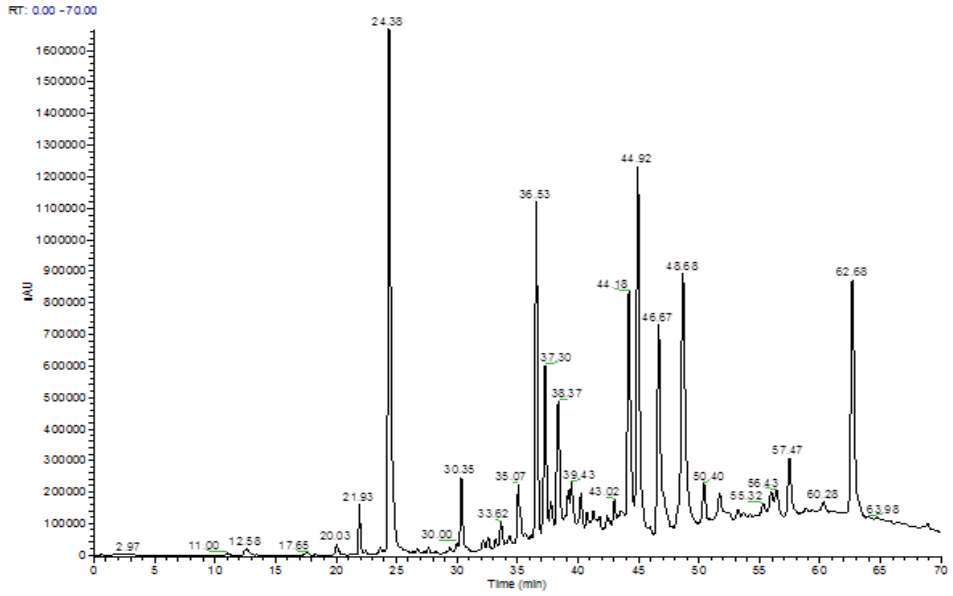

Figure 8. Representative chromatogram of mango Keitt polyphenols (Mango Extract A) at 280 nm

Mango extract A was tested in MCF-12A to determine its potential toxicity in non-cancer breast cells. A screening of different mango Keitt polyphenol concentrations (2.5-80 µg GAE/ml) revealed the growth inhibition of non-cancer breast cells by ~ 40% when mango Keitt polyphenol extract was applied at 2.5 µg GAE/ml. Higher doses showed a greater cell growth inhibition (~60%) (Appendix A). The ability of tannins to bind and precipitate proteins may explain the growth inhibitory effects exerted by mango Keitt polyphenols extract in MCF-12A non-cancer breast cells. Hydrolyzable tannins (gallotannins) consist of galloyl groups which have hydrophobic sites capable of interacting with aliphatic side chains of aminoacids through hydrophobic association to form complexes (*107*). The binding affinity property is dependent on the molecular weight of gallotannins and is enhanced by the addition of galloyl groups reaching a

maximum in the flexible disk-like structure of penta-O-galloyl-D-glucose (*107*). In addition, it is important to mention the role of pectinase in the polyphenol extraction. Pectinase is an enzyme that breaks down pectin (polysaccharide found in plant cell wall); therefore it is going to induce the release of polyphenol compounds leading to higher concentrations of these.

A second polyphenol extraction was performed to overcome the growth inhibitory effects describes previously. Mango Extract B was obtained following the same polyphenol extraction; however, the pH was slightly higher (2.98) than mango extract A (2.75) and pectinase was not used in the polyphenol extraction. In this study, the main polyphenolic compounds identified in mango Keitt pulp were gallic acid, hydroxybenzoic acid hexoside, and hydrolysable tannins such as mono-galloylglucoside and many others with different degree of galloylation ranging from penta-O-galloylglucoside to nona-O-galloylglucoside (Figure 9, Table 4). Similar results were reported by previous studies (*40, 44, 108*). Mangiferin was not detected in mango Keitt pulp. It is important to mention that mangiferin is a water soluble xanthone which is commonly found in the mango peel, therefore it was not detected since the polyphenol extraction was performed using the mango pulp. In addition, the content of mangiferin varies depending on several factors, including the cultivar. For instance, mangiferin was not detected at all in pulps of some of the 14 mango varieties from Africa, Asia, Australia and South America (*109*). In addition, it has been reported that factors such as the soil, ripening stage, harvest time and storage conditions are considered to affect the polyphenol contents of fruits, leading to a variability of the bioactive compounds (*110*).

The total C_{18} polyphenolic content of mango Keitt variety assessed by the Folin-Ciocalteu assay as well as the antioxidant capacity is shown in Table 3.

Table 3. Total C_{18} polyphenol content and antioxidant activity of mango Keitt extract

Mango Variety	Total C_{18} polyphenolic content (mg of GAE/gr. of pulp)	Antioxidant activity (µmol of TE/gr. of pulp)
Keitt	77.7	19.2

The polyphenolic content found in mango Keitt extract was 77.7 mg of GAE/gr. of pulp. Previous studies have reported a higher polyphenolic content in mango pulp (*40*). It should be noted that the polyphenolic content of mango Keitt extract comes from a C_{18} cartridge which can bind polar molecules such as sugars and vitamin C. The absence of these molecules in the final crude extract may have contributed to low polyphenolic content since any reducing agent such as sugars and vitamin C can react with the Folin-Ciocalteu method. Moreover, some factors are indicated to affect the polyphenol composition such as fruit ripening and method of extraction which in this study may have also affected the polyphenol content in the final crude extract.

Individually, most of mangoes' polyphenols contribute to their total antioxidant capacity. The antioxidant activity of mango Keitt extract was 19.2 µmol of TE/gr. of pulp. Previous studies have reported greater value; however tannins, in general, show high antioxidant potential due to their high molecular weight and high degree of

hydroxylation of aromatic rings (*111*). In addition, Hagerman et al. (*112*) suggested that tannins may be much more potent antioxidants than simple monomeric phenolic compounds. Since hydrolysable tannins are one of the major polyphenol compounds present in mango Keitt pulp, the antioxidant activity may be dependent on the many hydroxyl groups as well as the degree of polymerization; conferring them a better peroxyl radical scavenging capacity which may be presumably shown at even low concentrations. The ability of tannins to bind protein may have caused a precipitation of the proteins present in culture media, thus non-cancer breast cells were not able to proliferate normally.

Cell culture analyses were assessed using mango extract B since a preliminary screening showed no cytotoxic or inhibitory effects in the growth of MCF-12A non cancer cells. Apparently slightly differences in pH, as well as the method of extraction may have a significant impact in the mango polyphenolic composition and its antioxidant activity.

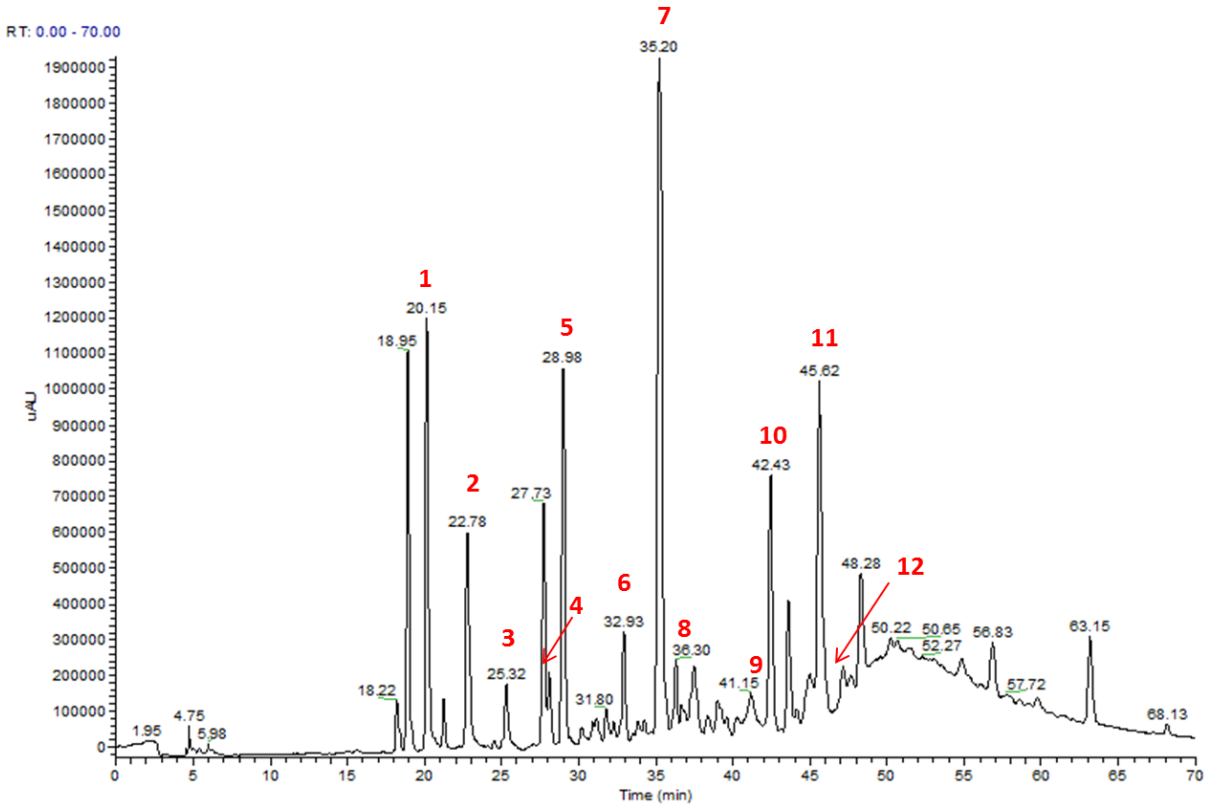

Figure 9. Representative chromatogram of mango Keitt polyphenols (Mango Extract B) at 280 nm

Table 4. Tentative characterization of mango Keitt polyphenols

Peak No	RT (min)	λ_{max} (nm)	Compound	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Concentration (mg GAE/L)
1	20.15	278	Mono galloyl-glucoside	331.1	271.1, 169.2, 211.1	27.29
2	22.78	271	Gallic Acid	169.2	125.2	16.62
3	25.32	270	Mono-Galloyl di-glucoside	493.2	313.10, 271.15, 331.09	3.05
4	28.09	270	Mono galloyl glucoside	331.1	169.2	4.50
5	28.98	260	hydroxybenzoic acid hexoside	299.1	1373.1, 179.0, 239.1, 208.9	52.86
6	32.93	290	Coumaric acid hexoside	324.79	163.12	3.30
7	35.2	266	Dihydrophaseic acid	443.1	237	69.79
8	36.3	314	Coumaric acid hexoside	325	145.3, 187.2, **163.2**, 265.1, 119.3	1.96
9	41.15	252, 379	Sinapic acid hexoside	385.02	223	
10	42.43	266	Dihydrophaseic acid	443.18	425.35, 237.13, 130.8	19.92
11	44.92	278	Penta-galloyl glucoside	939.14	769.10, 787.1, 617.14	29.54
12	47.15	278	Hexa-galloyl glucoside	1090.95	939	1.98

Effects of mango Keitt polyphenol extract (ME) on MCF-12A non cancer breast cell growth

The cell growth inhibition of ME was investigated in MCF-12A non-cancer breast cells after ME treatment for 48 h. Different concentrations of ME (2.5 – 80 μg GAE/ml) were tested in order to determine its potential toxicity. Results are indicating that when ME was applied at a dose range of 2.5-10 μg GAE/ml, MCF-12A cell growth was not significantly inhibited, thus at 10 μg GAE/ml, the percentage of cell growth inhibition was ~ 20% compared to the control. Similar results were reported in a study indicating that a polyphenolic extract from mango Ataulfo did not inhibit significantly the growth of CCD18Co colon myofibroblasts cells since the percentage of growth inhibition was only 13±6% even at the highest concentration of mango extract tested (10 μg/ml) (*40*). In addition, as a comparison to another tannin-rich fruit, a pomegranate extract (2.5-25 μg/ml) showed no significant effects in cell viability when applied to non-cancer MCF-10F and MCF-12F breast cells (*63*). Results are also indicating that higher doses of ME (20 – 80 μg GAE/ml) significantly inhibited MCF-12A cell growth since the percentage of cell growth inhibition was up to 50% (Figure 10). For this reason, further analyses were conducted using a dose range of 2.5 – 10 μg GAE/ml of ME.

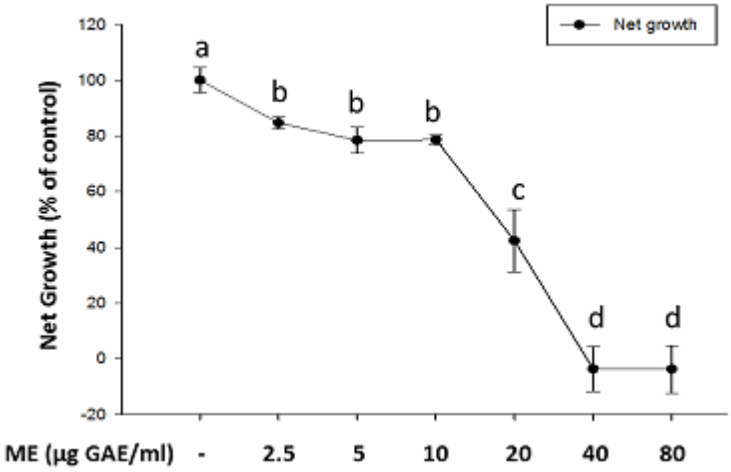

Figure 10. Cell proliferation of MCF-12A non-cancer breast cells treated with mango Keitt extract. Cells were treated with different concentrations of mango extract and cell growth was assessed after 48 h of incubation. Values are means SE (n=3). Different letters indicate significance at $p < 0.05$.

Effects of mango Keitt polyphenol extract (ME) on the generation of reactive oxygen species

Reactive Oxygen Species (ROS) are produced as a natural metabolism of oxygen and they are usually present in aerobic cells in balance with biochemical antioxidants (*22*). An imbalance in ROS production and elimination has been reported to cause deleterious effects such as structural changes in DNA as well as alterations in cytoplasmic and nuclear signal transduction pathways (*22, 113*). In addition, alterations in DNA caused by ROS have been indicated as one of the major causes of cancer (*22*). Considerable evidence has shown that ROS are likely to be involved between inflammation and cancer since tumor promoters are able to recruit inflammatory cells and induce them to produce ROS (*113*). Based on this premise, the generation of

reactive oxygen species was investigated after MCF-12A non cancer breast cells were challenged with tumor necrosis factor alpha (TNF-α). Results are indicating that ME reduced the generation of intracellular ROS compared to the positive control; however there were no significant differences between the different concentrations of ME tested (Figure 11). Similar results were reported by Pourahmad et al. (*114*) who evaluated the antioxidant properties of a mango extract (20-50 ug/ml) in rat hepatocytes against ROS formation. Even though no significant differences were found between mango extract treatments, mango decreased the production of ROS; suggesting its potential ability to protect the liver against the harmful effects of ROS. Another study reported that a mango peel extract protected rat erythrocytes against oxidative stress induced by H_2O_2 (*115*).

Overall, it has been reported that polyphenols present in mango show protection against oxidative damage due to their antioxidant activity (*116, 117*). Results in this study are indicating that in an inflammatory microenvironment, the antioxidant and radical scavenging properties of ME may cross the cell membrane and cope with the intracellular ROS formation as previously reported (*114*). Thus, the effects of ME against ROS formation and oxidative stress may be attributed to its ability to scavenge free radicals likely based on its antioxidant capacity which can be attributed to the presence of hydrolysable tannins. In fact, hydrolysable tannins along with condensed tannins have shown better peroxyl radical quenching capacity than simple phenols due to the presence of many hydroxyl groups and degree of polymerization (*112, 118, 119*).

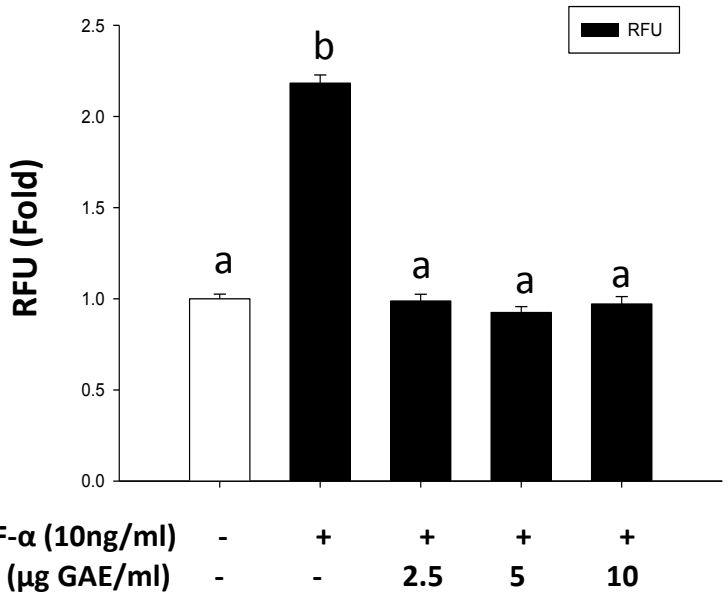

Figure 11. TNF-α induced generation of reactive oxygen species (ROS) in MCF-12A non cancer breast cells treated with ME. ROS generation was detected using 2,7-dichlorofluorescein diacetate (DCFH-DA). Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$.

Consequently, the findings in this study are suggesting the possible protective role of ME against the deleterious effects of ROS and oxidative stress. In addition, the ability of ME to scavenge free radicals and to attenuate oxidative stress was demonstrated indicating the potential role that mango polyphenols may have in protecting non-cancer breast cells against ROS production.

Estrogen, glucose and tumor necrosis factor alpha (TNF-α) induced inflammation in MCF-12A cells

Increased levels of circulating estradiol and glucose have been linked to a higher incidence of breast cancer (*120*). Estrogen is a hormone that acts as a mitogen promoting cell proliferation in normal breast tissue and breast carcinoma. In addition, estrogen is also capable of activating multiple signaling pathways leading to proliferation of human breast cancer cell lines in vitro (*100*). Glucose and other factors associated with glucose metabolism, such as insulin and insulin-like growth factors (IGFs) may contribute to breast cancer development as well (*121*). It has been reported that increased levels of glucose may fuel estrogen production leading to an increase incidence of estrogen positive breast cancer in obese postmenopausal women (*122, 123*).

As a model to evaluate the potential effects of ME in an inflammatory microenvironment, MCF-12A non-cancer breast cells were stimulated with estrogen (10nM and 25 nM), glucose (25mM) and a combination of glucose (25 mM) and estrogen (10nM). In addition tumor necrosis factor alpha (TNF-α) was also tested at a concentration of 10 ng/ml since it is considered a master switch for inflammation. It has been reported that estrogen is a potent mitogen and may act synergistically with glucose related factors to trigger signal transduction pathways; however, findings in this study are showing that after 3 h. of incubation, neither estrogen and glucose nor the combination of both induced inflammation in MCF-12A non-cancer breast cells (Figure 12). In general, there were no significant changes in the expression of inflammatory markers such as NF-κB, IL-6, IL-1β and TNF-α which have been implicated in obesity-

induced inflammation. In addition, the levels of these pro-inflammatory cytokines have also been related in breast inflammation and cancer. In contrast, when MCF-12 non-cancer breast cells were stimulated with TNF-α for 3 h., there was a significant increase in the gene expression of inflammatory genes such as NF-κB, IL-6, IL-1β and TNF-α (Figure 12). For these reasons, further analyses were conducted using TNF- α (10 ng/ml).

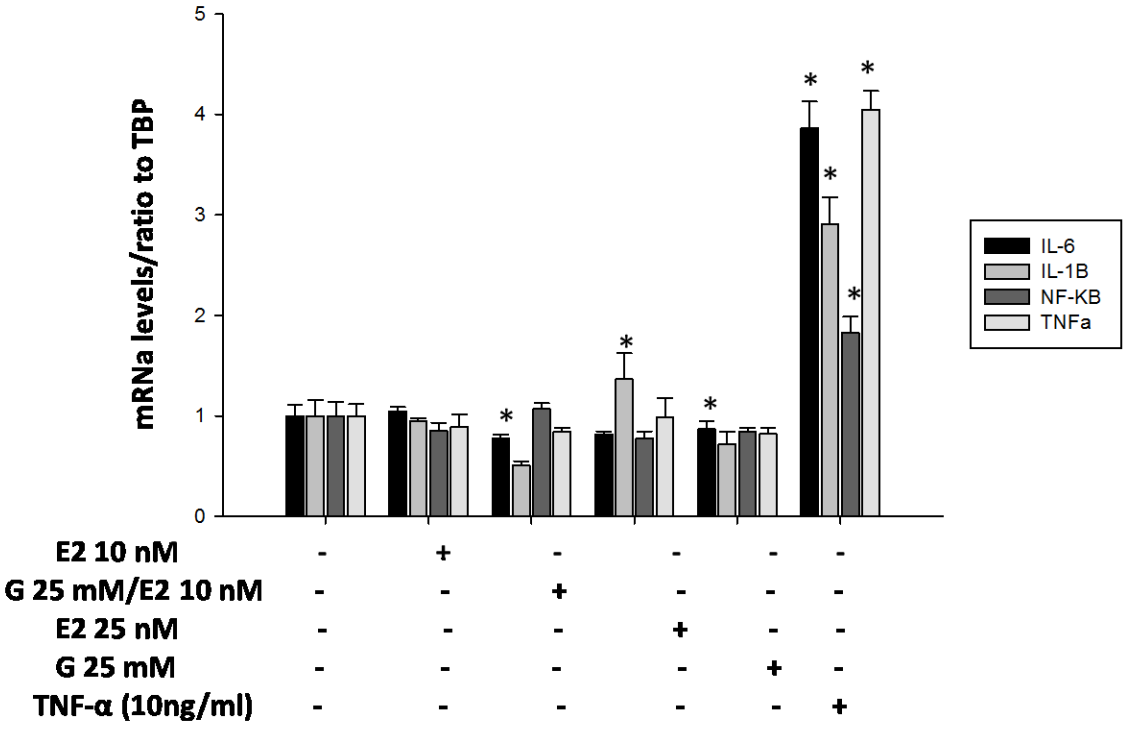

Figure 12. Estrogen (E), Glucose (G) and TNF-α induced inflammation in MCF-12A non-cancer breast cells. Inflammation was induced for 3 h. Each experiment was performed at least three times and results are expressed as means ± SE. Significant difference is denoted by * at $p < 0.05$.

Effects of ME in the tumor necrosis factor alpha (TNF-α) induced inflammation

An inflammatory microenvironment in the breast may be both an intermediary of several risk factors linked with breast cancer as well as a player in the development of aggressive breast tumors (*4*). Therefore, inflammation might be considered as an anti-cancer therapeutic opportunity (*70*). Based on this premise, the effects of ME on TNF-α induced inflammation were assessed in MCF-12A non-cancer breast cells as a model to investigate its potential role in the modulation of inflammatory markers. The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) was used to induce inflammation in MCF-12A non-cancer breast cells since it is considered as one of the major mediators of inflammation triggering the expression of several pro-inflammatory cytokines (*124*). Results shown in Figure 13 are indicating that at the mRNA level, the TNF-α induced expression of IL-6, IL-1β, NF-κB and VCAM was significantly down-regulated by up to 0.69, 0.7, 1.38 and 2.41- fold of TNF-α challenged cells respectively when ME was applied at 10 μg GAE/ml. Moreover, the protein expression of phosphorylated NF-κB was also decreased by ME at even lower concentrations (1.25-5 μg GAE/ml) (Figure 14); however full length NF-κB protein was not decreased by ME.

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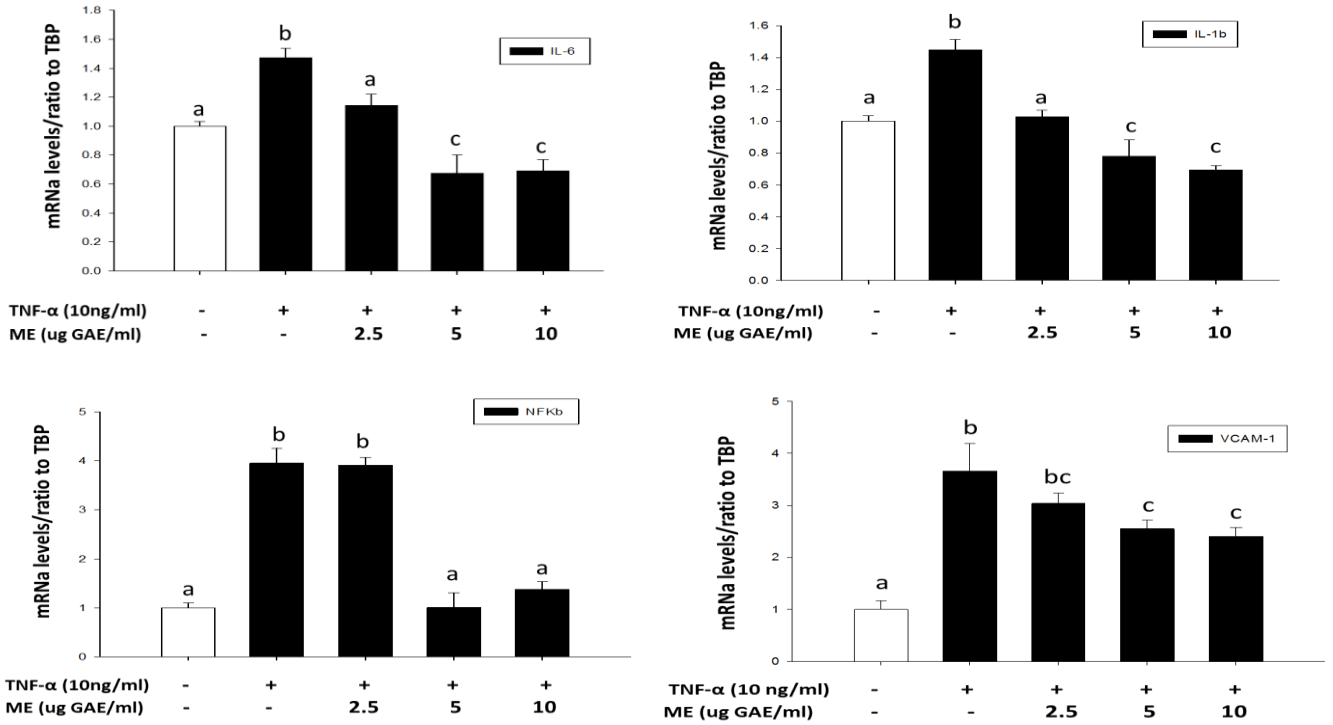

Figure 13. Gene expression of IL-6, IL-1β, NF- κB and VCAM in MCF-12A non-cancer breast cells after 24 h incubation with mango Keitt extract (ME) and TNF-α challenge for 3 h. Gene expression was analyzed by qRT-PCR as a ratio to TATA binding protein (TBP) mRNA. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$.

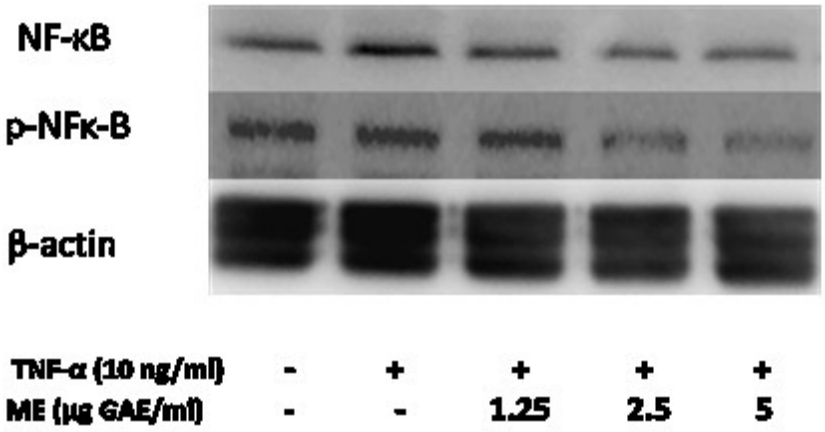

Figure 14. NF-κB and pNF-κB protein expression. MCF-12A non-cancer breast cells were treated with different concentration of ME for 24 h and protein expression was analyzed by Western Blot. Relative protein expression was normalized to β-actin.

The hallmarks of cancer-related inflammation comprise the incidence of inflammatory cells and inflammatory mediators (*5*) such as TNF-α which is considered a master switch for inflammation to cancer (*125*). Among the members of the TNF-α superfamily, the inflammatory cytokines such as interlukin-6 (IL-6) and interlukin 1 beta (IL-1β) have been linked to tumorigenesis suggesting that inflammation is related with cancer development (*68*). Additionally, IL-6 and IL-1β are considered critical mediators of the inflammatory response (*126*)**.** Vascular cell adhesion molecule-1 (VCAM) is up-regulated following inflammation and its implication in breast tumor progression has been proposed (*127*). The expression of these genes is orchestrated by NF-κB which is considered a master key regulator in transforming inflammation into cancers (*128*)

In this study, ME down-regulated the gene expression of NF-κB and its downstream targets involved in inflammation. Mango is a fruit rich in phenolic acids (i.e gallic acid) and hydrolysable tannins (i.e gallotannins) which have been reported to exert

multiple important biological properties. Indeed, anti-inflammatory activity is one of the most important effects attributed to phenolic acids and tannins (*111, 129*); however, the mechanism of action exerted by these polyphenols compounds has not been elucidated yet due to the complex character of inflammatory processes (*111*). This is the first study reporting the modulation of pro-inflammatory genes by mango polyphenols in MCF-12A non-cancer breast cells. However, one of the main polyphenol compounds identified in mango such as gallotannins have shown to inhibit the *in vitro* NF-κB activation and its related downstream genes in human monocytic cells stimulated with TNF-α (*111*).

Overall, the findings in this study are suggesting the capacity of mango Keitt polyphenols to modulate inflammatory genes activated in non-cancer breast cells. Therefore, mango Keitt polyphenols hold a promising anti-inflammatory therapeutic strategy for preventing and/or controlling inflammation and associated chronic diseases, in particular breast cancer

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Modulation of the PI3K/AKT/mTOR pathway by ME

The phosphoinositide-3-kinase (PI3K)/Akt signaling pathway plays a central role in the cellular response to growth factors and controls key cellular functions such as growth, metabolism, migration, apoptosis and survival (*76, 130, 131*). This signaling pathway is activated by tyrosine kinase growth factor receptors such as insulin growth factor-1 receptor (IGF-1R), among others (*76*). In order to evaluate the effects of mango polyphenols in the modulation of the PI3K/AKT/mTOR pathway, MCF-12A non-cancer

breast cells were stimulated with IGF-1 (20 and 50 ng/ml) since it is a potent mitogen to many different cell types *in vitro* (*132*). After 3 h. of incubation with IGF-1 at two different concentrations, no significant changes were found between the negative and positive control in MCF-12A non-cancer breast cells indicating that inflammation and/or the activation of the PI3K/AKT pathway was not produced (Figure 15). In contrast, when MCF-12A cells were induced with TNF-α (10ng/ml), the PI3K/AKT pathway was significantly induced as shown in Figure 16. These results are in accordance with previous reports indicating that AKT is involved in the activation of NF-κB by TNF-α which in turns activates PI3K and its downstream target AKT (*133*).

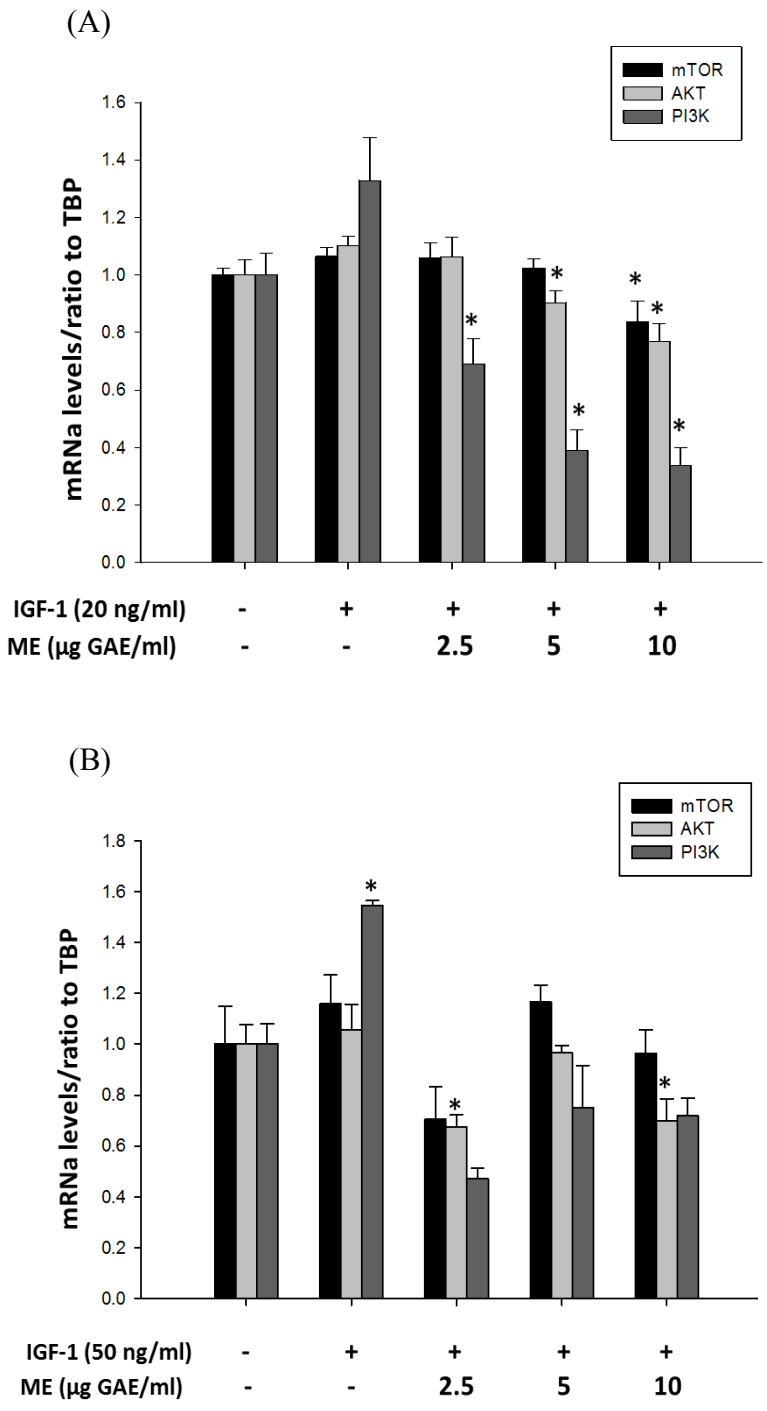

Figure 15. Gene expression of PI3K, AKT and mTOR. MCF-12A non-cancer breast cells were induced with (A) IGF-1 20 ng/ml and (B) IGF-1 50ng/ml for 3 hr. Each experiment was performed at least three times and results are expressed as means ± SE. Significant difference is denoted by * at $p < 0.05$

Since the PI3K/AKT pathway was stimulated when MCF-12A cells were challenged with TNF-α for 3 h, different concentrations of ME were tested (2.5-10 μg GAE/ml) to determine its potential anti-inflammatory effects by inhibiting the activation of the PI3K/AKT signaling pathway and its related downstream targets such as the mammalian target of rapamycin (mTOR). Results are showing that PI3K, AKT and mTOR were up-regulated by TNF-α by 1.74, 1.73 and 1.28-fold respectively; however, this effect was reverted by ME since at 10 μg GAE/ml, the gene expression of PI3K was significantly reduced to 1.16-fold. Moreover, the phosphorylation of AKT and mTOR was significantly down-regulated to 0.75 and 0.62-fold of TNF-α challenged cells, respectively (Figure 16). In the case of mTOR, ME decreased the expression of this gene in a concentration dependent manner.

These results were accompanied by a decrease in the total and phosphorylated-AKT (p-AKT) protein expression analyzed by multiplex bead analysis (Figure 17 A) In contrast, the total protein expression of mTOR was not significantly inhibited whereas its phosphorylated form showed opposite results since ME polyphenol treatment down-regulated the expression of p-mTOR in a dose dependent manner (Figure 17 B). The activation of mTOR induces protein synthesis by phosphorylating the ribosomal protein S6 kinase or p70S6K. Consequently, p70S6K phosphorylates the S6 ribosomal protein (RPS6) to promote initiation of mRNA translation. Interestingly, ME polyphenols decreased the protein expression of total p70S6K and its phosphorylated form in a concentration dependent manner. In addition, a similar trend was shown in the protein

expression of RPS6 suggesting the involvement of ME in protein synthesis (Figure 17 C,D).

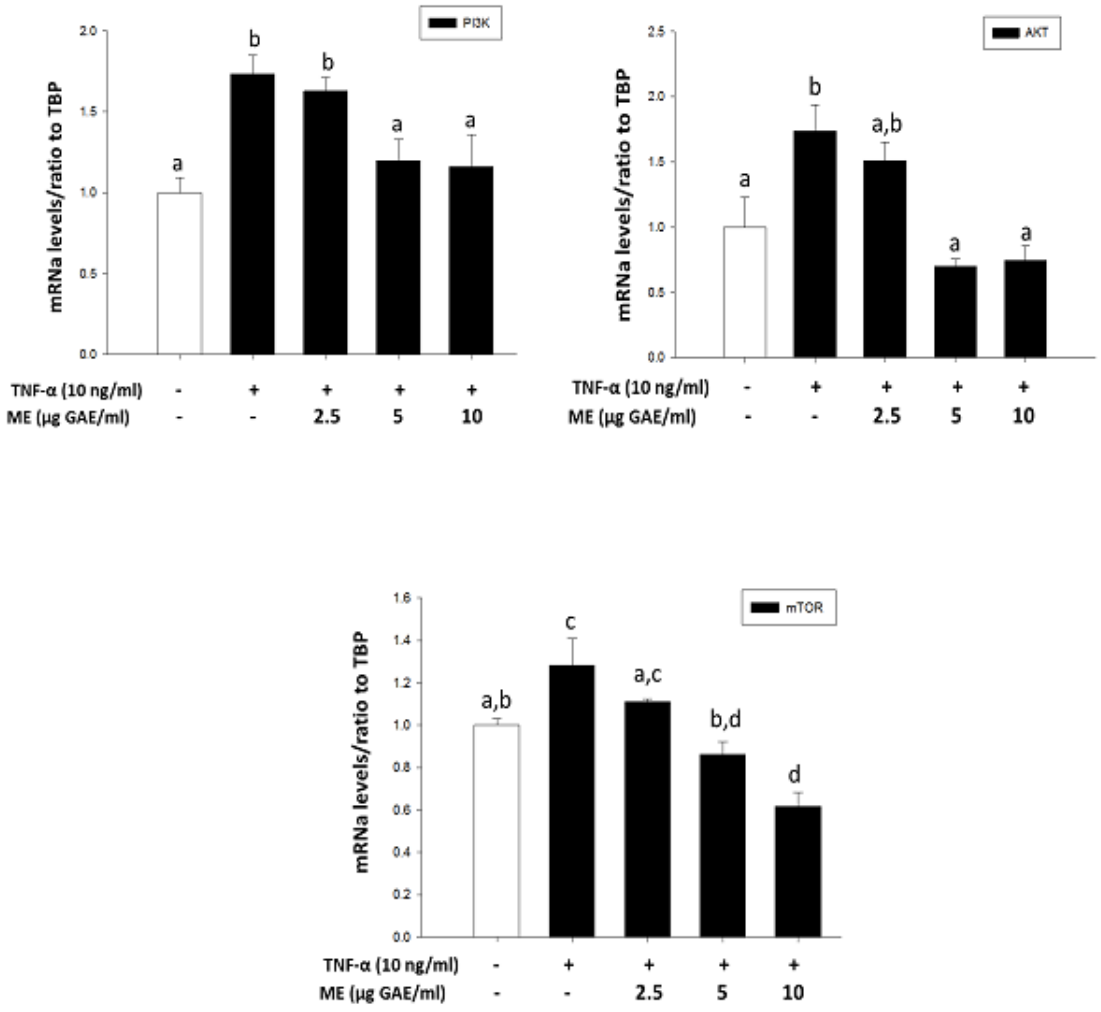

Figure 16. Gene expression of PI3K, AKT and mTOR in MCF-12A cells after 24 h of incubation with ME and TNF-α challenge for 3 h. Gene expression was analyzed by qRT-PCR as a ratio to TATA binding protein (TBP). Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$.

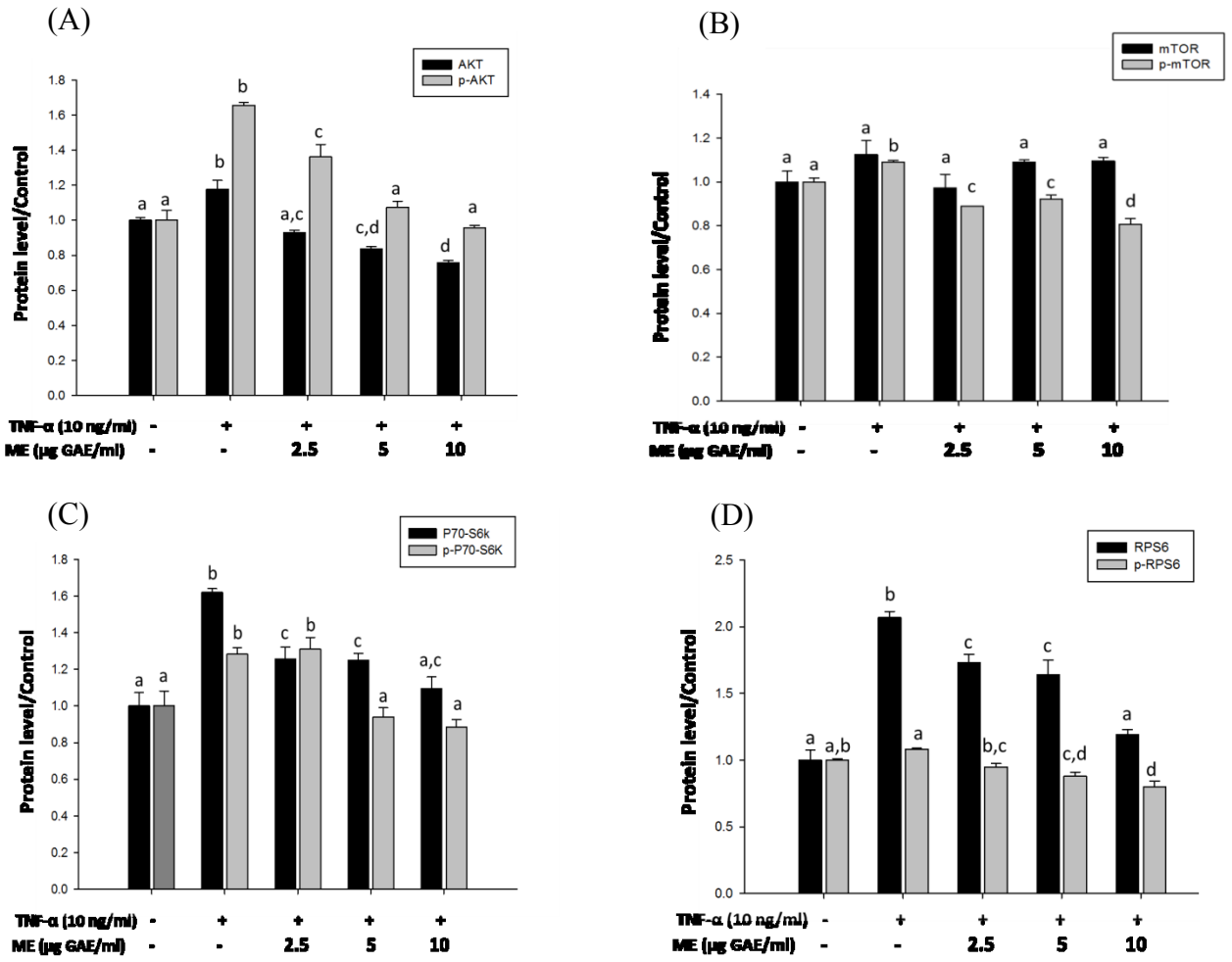

Figure 17. Protein expression of (A) AKT, p-AKT; (B) mTOR, p-mTOR; (C) P70S6K, p-P70S6K and (D) RPS6, p-RPS6 analyzed by multiplex bead analysis as described in Materials and Methods. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$.

Polyphenols present in natural plant extracts appear to target the TNF-α activation of PI3K/AKT signaling pathway. For instance, a cocoa polyphenol extract inhibited the TNF-α induced phosphorylation of Akt and suppressed the PI3K activity in mouse epidermal cells (*86*). Individual polyphenols found in mango have been reported to suppress the PI3K/AKT pathway. For instance, gallic acid and gallotannins inhibited the activation of PI3K/AKT and related downstream targets such as mTOR and NF-κB suggesting their potential anti-inflammatory properties; however, most of these studies were conducted in several cancer cells *in vitro (134-136).* Even though the association between the PI3K cascade and cancer is strong, evidence indicates its importance in other conditions, notably inflammatory diseases. The findings in this study are indicating the potential anti-inflammatory properties of ME polyphenols in non-cancer breast cells by suppressing the PI3K/AKT cascade accompanied by a decreased expression of NF-κB and mTOR and related downstream genes which are direct targets of the PI3K cascade. The multiple biological properties of single compounds present in mango such as gallic acid and gallotannins are likely to have contributed to the anti-inflammatory properties in MCF-12A non-cancer breast cells. Therefore, mango polyphenols present in mango variety Keitt may be considered as a novel and promising inhibitors of the PI3K/AKT pathway in an inflamed breast environment suggesting a possible prevention of breast cancer development.

Effects of ME on the gene expression of miRNA-126

MicroRNAs (miRNAs) are small non-coding RNAs able to regulate crucial cell processes such as proliferation, differentiation and apoptosis (*137*). Among the hundreds of miRNAs identified, miRNA-126 has been intensively studied and conferred significant roles in different physiological and pathological processes, including inflammation and cancer (*93*). Previous studies have reported the role of miRNA-126 in non-cancer and colon cancer cells; however, research indicating the anti-inflammatory properties of natural plant extracts in regulating the expression of miRNA-126 in non-cancer breast cells is limited. Therefore, MCF-12A non-cancer breast cells were treated with different concentrations of ME polyphenols (2.5-10 μg GAE/ml) for 24 h. and induced inflammation with TNF-α (10 ng/ml) since during inflammation the expression pattern of noncoding miRNAs changes dramatically (*93*).

Results are showing that ME up-regulated the expression of miRNA-126 in a concentration dependent manner. Moreover, TNF-α significantly decreased the expression of miRNA-126; however, at 10 μg GAE/ml, ME polyphenols significantly reversed this effect by inducing the expression of miRNA-126 up to 1.4 fold of TNF-α-challenged cells (Figure 18).

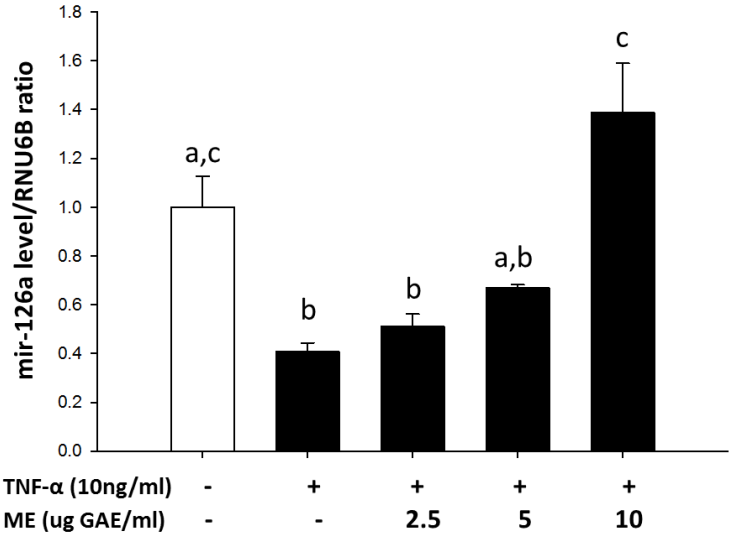

Figure 18. miRNA-126 expression of MCF-12A cells treated with mango Keitt extract (ME) after 24 h and analyzed by qRT-PCR as a ratio to RNU6B miRNA. Values are mean ± SE (n=3). Different letters indicate significance at $p < 0.05$

In order to confirm the up-regulation of miRNA-126 gene expression by ME polyphenols, MCF-12A non-cancer breast cells were transfected with a specific antagomir (Ant.) for miRNA-126. The antagomir for miRNA-126 decreased the gene expression of miRNA-126 whereas ME polyphenols significantly reversed this effect, thus at 10 μg GAE/ml of ME, the levels of miRNA-126 were significantly increased by up to 0.65-fold of TNF-α challenged cells (Figure 19). miRNA-126 can modulate the PI3K signaling pathway by targeting the PI3K regulatory subunit beta (p85β) in colon and lung cancer cells (*94, 138*). To evaluate the potential modulation of PI3K (p85β) by miRNA-126 in an inflamed microenvironment, MCF-12A non-cancer breast cells were induced inflammation with TNF-α (10ng/ml) and transfected with miRNA-126 antagomir. Results are showing that after transfection, the mRNA expression of PI3K

was increased; however, this effect was significantly reversed by ME polyphenols at 10 μg GAE/ml, even when MCF-12A non-cancer breast cells were challenged with TNF-α, since the expression of PI3K p85β subunit was reduced to 0.42-fold of TNF-α challenged cells (Figure 20).

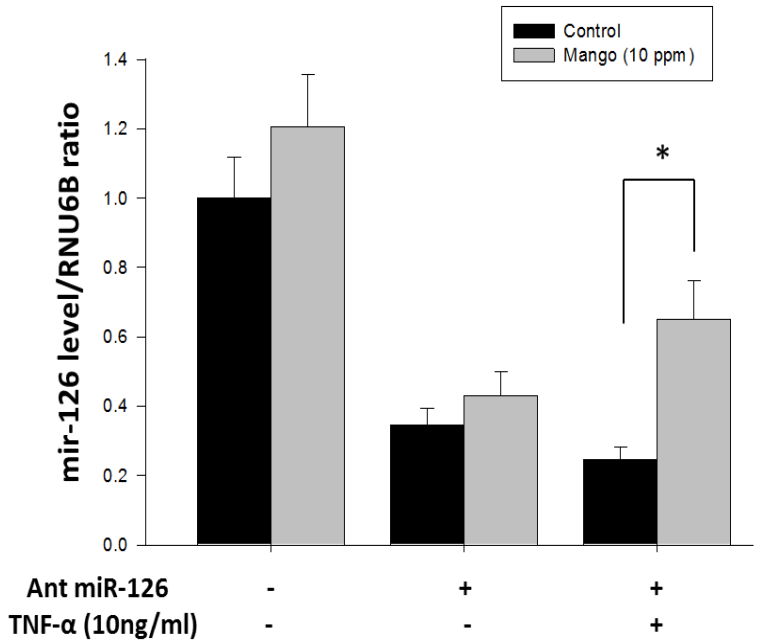

Figure 19. Expression of miRNA-126 after transfection with antagomir miRNA-126 in MCF-12A cells. Cells were treated with ME (10 ug GAE/ml) before TNF-a challenge (10ng/ml) and relative miRNA levels were analyzed by qRT-PCR as described in Materials and Methods. Bars represent means ± SEM, n = 3. Statistically significance difference is denoted by * (T-test, $p < 0.05$).

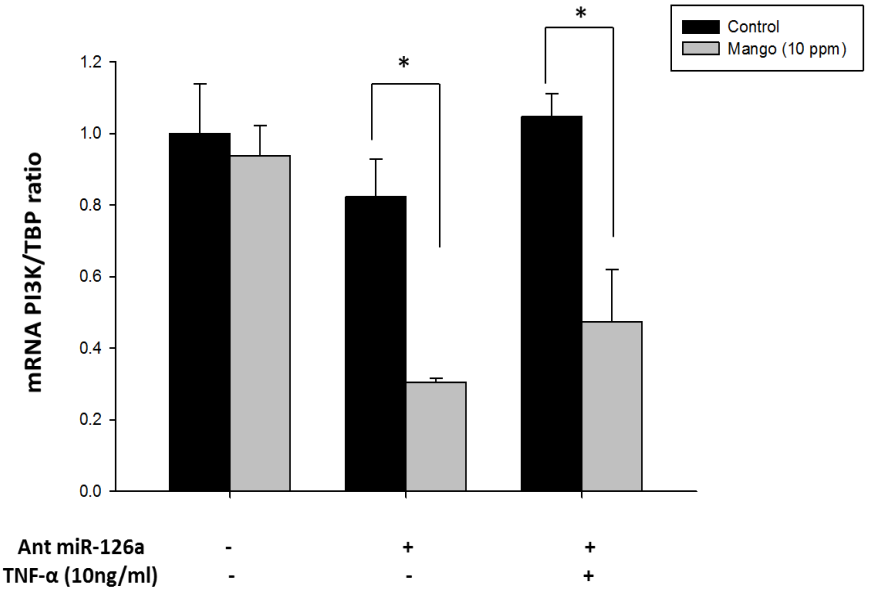

Figure 20. Expression of PI3K after transfection with antagomir miRNA-126 in MCF-12A cells. Cells were treated with ME (10 µg GAE/ml) before TNF-α challenge (10 ng/ml) and relative mRNA levels were analyzed by qRT-PCR as described in Materials and Methods. Bars represent means ± SEM, n = 3. Statistically significance difference is denoted by * (T-test, $p < 0.05$).

Since the expression of miRNA-126 is frequently lost in several types of cancer, the role of polyphenols in the up-regulation of miRNA-126 gene expression has been previously reported. In general, polyphenols isolated from different fruit sources such as pomegranate; acai and grape have shown a significant impact in the up-regulation of miRNA-126 suggesting their potential anti-inflammatory and chemopreventive properties (*101, 139*). Findings in this study lead to assume that the miRNA-126 gene expression modulation may be one of the underlying mechanisms that contribute to the anti-inflammatory effects of ME polyphenols in MCF-12A non-cancer breast cells as TNF-α inhibited miRNA-126 gene expression but ME polyphenols reversed such effect

in a concentration dependent manner up to basal levels (control cells without TNF-α induction). In addition, after transfection with a specific antagomir for miRNA-126, the gene expression of this miRNA was suppressed; however, the effects of the antagomir were reversed by ME polyphenols at 10 μg GAE/ml, confirming the regulation of miRNA-126 gene expression as a potential mechanism of the anti-inflammatory properties of ME polyphenols.

Results are showing an overexpression of PI3K (p85β) subunit after transfection with antagomir miRNA-126; however, ME polyphenols counteracted such effect. Even more, a similar trend was found in transfected cells exposed to TNF-α, in which the PI3K overexpression was significantly down-regulated by ME polyphenols. Wang et al. (*94*) suggested the PI3K/AKT pathway as a downstream target of miRNA-126. Moreover, Guo et al. (*138*) reported that miRNA-126 can modulate the PI3K cascade by targeting the PI3K p85β subunit, supporting the findings in this study.

The polyphenols present in mango fruit are likely to play an important role in the regulation of miRNA-126, which effects are mediated by the PI3K/AKT pathway. This is the first study in which mango Keitt polyphenols are reported to exert anti-inflammatory properties in MCF-12A non-cancer breast cells by inducing an overexpression of the tumor suppressor miRNA-126 which targets the PI3K cascade.

Overall, findings in this study are suggesting the capacity of mango Keitt polyphenols to regulate the gene expression of small non-coding RNAs involved in complex and detrimental processes such as inflammation in non-cancer breast cells.

Conclusion

In conclusion, mango Keitt polyphenols were able to attenuate the process of inflammation in non-cancer breast cells by inhibiting the activation of NF-κB and related downstream targets such as IL-6, IL-1β and VCAM-1. The suppression of the PI3K/AKT/mTOR pathway as well as an induced overexpression of miRNA-126 were identified as possible underlying mechanisms that contribute to the overall anti-inflammatory effects of mango Keitt polyphenols. These results suggest that mango Keitt could be considered as a novel and cheap source of dietary polyphenols capable of inhibit the detrimental effects of cancer-related inflammation in mammary cells.

CHAPTER IV

MANGO KEITT POLYPHENOLS EXERTS ANTI-INFLAMMATORY AND CYTOTOXIC PROPERTIES IN MDA-MB231 BREAST CANCER CELLS BY TARGETING miRNA-21

Summary

Mangoes are considered a novel source of bioactive compounds containing a mixture of polyphenols such as phenolic acids, flavonoids, hydrolysable tannins and carotenoids which have been related not only to enhance the general well-being but also to prevent the onset of degenerative diseases, including cancer. Several studies have demonstrated that mango polyphenols may exert antioxidant, anti-inflammatory and anti-carcinogenic activities in colon and breast cancer cells; however their mechanism of action has not been extensively reported. The main objective of this study was to evaluate the anti-inflammatory and cytotoxic effects of mango Keitt polyphenol extract (ME) in MDA-MB231 breast cancer cells through the modulation of apoptotic genes and the regulation of the PI3K/AKT/mTOR signaling pathway. In addition, the potential involvement of miRNA-21 as an underlying mechanism of the anti-carcinogenic effects of mango Keitt polyphenols was investigated. ME exerted cytotoxic effects by inhibiting the cell growth of MD-MB231 breast cancer cells within a concentration range of 1.25-10 μg GAE/ml. These results were accompanied by a significant regulation of pro and anti-apoptotic biomarkers and a decreased production of reactive oxygen species. A significant decrease in the gene and protein expression of NF-κB and VEGF was also

observed after ME treatment suggesting the anti-inflammatory and angiogenic properties of mango Keitt polyphenols. ME also suppressed the activation of the PI3K/AKT pathway by regulating the gene and protein expression of PI3K, AKT and related downstream targets such as mTOR and HIF-1α. As a result, ME (1.25-10 μg GAE/ml) supressed the gene expression of oncomir miRNA-21 whereas its main gene target PTEN was significantly induced after ME treatment. The effects of the specific antagomir for miRNA-21 as well as the PTEN gene expression in knockout breast cancer cells were partially reversed after ME treatment. In summary, the anti-inflammatory and cytotoxic effects of mango Keitt polyphenols may be partly due to the supression of the PI3K/AKT/mTOR signaling pathway with relevant regulation of the miRNA-21-PTEN axis.

Introduction

Due to its diversity in size, color and flavor, mangoes (*Mangifera indica* L.) are utilized for the production of a great variety of products placing them as one of the tropical fruits most consumed worldwide. In addition, mangoes popularity has also been increasing due to its promising nutraceutical value due to presence of a mixture polyphenolic compounds such as phenolic acids, flavonoids, hydrolyzable tannins, and carotenoids identified in the edible part of the fruit. In fact, several studies have reported the antioxidant anti-carcinogenic effects of mango pulp polyphenols in breast, colon and leukemia cancer cells (*40, 49, 50*). In addition, mango sub-products such as the peel,

stem bark and kernel have demonstrated to contain great amount of polyphenolic compounds conferring them antioxidant and antiproliferative properties as well (*140*).

Dietary polyphenols have been tested *in vitro* and *in vivo* to determine their potential role in the prevention of several types of cancer; including breast cancer since it is one the leading causes of death from cancer among women worldwide. Diet seems to play a preventive and protective role in the prevention of this chronic disease because it has been estimated that 30-40% of cancer diseases can be prevented with a healthy lifestyle and diet (*53*). Consequently, a regular consumption of dietary polyphenols from fruit and vegetables sources has been associated with positive influences on health. It has been reported that polyphenols from mango exerted cytotoxic, antiproliferative and anti-tumor properties on breast cancer cells suggesting its potential use as a chemopreventive agent in the prevention of breast cancer. (*40, 141, 142*) However, their molecular mechanism of action needs to be further clarified for clinical applications.

The PI3K/AKT/mTOR pathway is altered in human cancers and is considered as an attractive therapeutic target in breast cancer since its aberrant expression controls and regulates several mechanisms such as cell proliferation, survival and anti-cancer drug resistance (*74, 76, 77*). Dietary polyphenols have called the attention of researchers looking for treatment strategies to suppress the PI3K/AKT pathway and its downstream effectors in breast cancer treatment. Indeed, previous studies have shown the capacity of polyphenol compounds to inactivate the PI3K pathway and downstream genes (*85, 87, 88*).

Among the micro-RNAs (miRNAs) identified in breast cancer, miRNA-21 seems to be key molecule implicated in the malignant profession of breast cancer since its overexpression has been correlated with an increased proliferation and tumor growth in breast cancer cells as wells as advance tumor stage, lymph node metastasis and poor survival of breast cancer patients (*90, 143*). The aberrant expression of miRNA-21 is associated with the inactivation of the tumor suppressor PTEN which is considered an important downstream target of miRNA-21 In addition, alterations in PTEN levels are related with dramatic changes in the PI3K/AKT pathway (*80, 96*). Nutritional factors can influence the expression of miRNAS, therefore dietary polyphenols may contribute to reduce the risk of chronic diseases by modulating the gene expression of miRNAs.

The goal of this study was to investigate the efficacy of mango Keitt polyphenol extract as an anti-inflammatory and cytotoxic agent in MDA-MB231 breast cancer cells by the modulation of apoptotic genes and the suppression of the PI3K/AKT/mTOR signaling pathway. The potential involvement of the miRNA-21-PTEN axis as an underlying mechanism of the anti-carcinogenic effects of mango Keitt polyphenols was also investigated.

Materials and Methods

Plant material

Mango (*Mangifera indica* L.), variety Keitt, was provided by the National Mango Board (U.S). This fruit was obtained at a green stage and was allowed to ripen at room temperature until a moderate ripe stage was reached. Fruit was manually peeled to remove skin and seed and only the pulp was used for polyphenol extraction. Pulp was diced, vacuum sealed and stored at -20 C until needed.

Cell line

MDA-MB231 breast epithelial cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 1% penicillin/streptomycin, 1% non-essential amino acids (10mM), 1% sodium pyruvate (100 mM) and 10% of fetal bovine serum (Invitrogen, Carlsbad, CA). Cells were maintained at 37 ºC with a humidified 5% CO2 atmosphere.

Chemicals, antibodies and reagents

Standards for HPLC-MS analysis were obtained from Sigma-Aldrich (St Louis, MI). The Folin-Ciocalteu reagent was purchased from MP biochemical, LLC (Solon, Ohio). 2'7'-dichlorofluorescein diacetate (DCFH-DA), dimethyl sulfoxide (DMSO) and Tumor Necrosis Factor alpha (TNF-α) were purchased from Sigma (St Louis, MI). Janus green was purchased from Fisher Scientific (Pittsburgh, PA). Bradford reagent was

obtained from BioRad (Hercules, CA), antibodies against NF-kB p65, phospho-NF-kB p65, VEGF, AKT, mTOR and β-actin were obtained from Cell Signaling Technology (Beverly, MA). PI3K (p85β) was purchased from Abcam (Cambridge, MA). All primers were purchased form Integrated DNA Technologies (San Diego, CA). MirVana TM extraction kit, reverse transcription (RT) and real-time PCR amplification kit were purchased from Applied Biosciences (Foster City, CA). miRNA-21 and antagomir for miRNA-21 were purchased from Dharmacon, Inc. (Lafayette, CO).

Mango polyphenols extraction

Mango pulp was extracted as previously described with slightly modifications (*40*). Briefly, mango pulp was thawed and homogenized in a blender and extracted in a ratio of 500g of pulp to 1.5 L of a solvent mixture (methanol:ethanol:acetone, 1:1:1) for 1 h. at room temperature. After 1 h. incubation with solvents and pectinase, solids were centrifuged at 3000 rpm for 10 minutes followed by filtration and extracted twice following the same procedure. The solvents were removed under reduced pressure at 40°C. Mango polyphenols were partitioned using a $20cm^3$ Waters C18 cartridge (Waters Corporation, Milfords, MA) previously conditioned with methanol 100% and acidified (0.01% HCl) water. Those compounds that were not adsorbed in the cartridge were extracted with ethyalcetate in a separatory funnel. The ethylacetate fraction and methanol phase from the C18 cartridges were combined and the solvents were removed under reduced pressure until dryness was reached. The dried extract was reconstituted in

DMSO (0.2%) for cell culture assays. A control with 0.2% DMSO was included in all assays.

Separations were made on an Acclaim™ C18 column (Bannockburn, Il), (250 x 4.6 mm, 5 μm) at room temperature. The mobile phase consisted of 0.1% formic acid in water (Phase A) and 0.1% water (Phase B). A gradient program at 0.4 mL/min initially ran Phase B at 0%, for 3 minutes, 21% Phase B in 20 min, from 21 to 35% Phase B in 30 min, and 35 to 49% Phase B in 50 min, 49% to 70% Phase B in 70 minutes before returning to initial conditions. Detection was at 280 and 360 nm for benzoic and cinnamic acids/flavonoids, respectively. Compounds were tentatively identified based on mass spectrometric analysis. This was performed on a Thermo Finnigan LCQ Deca XP Max ion trap mass spectrometer with an electrospray ionization probe in negative ion mode under the following conditions: sheath gas (N2), 40 units/min; auxiliary gas (N2), 5 units/min; spray voltage, 4.5 kV; capillary temperature, 300°C; capillary voltage, 7.0 V; tube lens offset, 40.0 V, source current at 80.0 μA.

Determination of total soluble phenolics

Total soluble phenolics were measured by the the Folin-Ciocalteu assay (*104*) at 726 nm. Gallic acid was used as a standard to quantify total soluble phenolics by linear regression. Results were expressed as mg of gallic acid equivalents (GAE) per gram of mango pulp.

Antioxidant capacity

Antioxidant capacity was assessed by the oxygen radical absorbance capacity (ORAC) assay using a BGM Labtech FLUOstar fluorescent microplate reader (485 nm excitation and 538 nm emission), as previously described (*105*). Results were expressed in μmol Trolox equivalents (TE) per gram of mango pulp.

Cell proliferation

Cells (1.5×10^4) were grown in a 24 well plate and incubated for 24 h to allow cell attachment. Cells were treated with different mango polyphenol concentrations (1.25 – 10 ug/ml). Cell proliferation was determined after 48 h incubation with mango extract using a cell counter (Z2 Series Beckman Coulter, Fullerton, CA). Cell counts were expressed as a percentage of control cells as previously described (*40*).

Generation of reactive oxygen species

Determination of reactive oxygen species was assessed as previously described (*105*) using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) assay with slightly modifications. MDA-MB231 breast cancer cells (1.5×10^4) were seeded in a black bottom 96 well plate and treated with different mango polyphenol concentrations (1.25 - 10 ug/ml) for 24 h. ROS production was detected using DCFH-DA (10uM) for 30 min at 37°C. Fluorescence signal was measured at 520 nm emission and 480 nm excitation in a FLUOstar Omega microplate reader (BMG Labtech Inc, Durhan, NC). After monitoring the fluorescence signal, cell counting was determined as described before (*106*). Briefly,

MCF-12A cells were washed twice with PBS and fixed with 100% methanol for 3 min. After removal of methanol, cells were stained with 1mg/ml Janus green for 3 min, washed twice with PBS and 50% methanol was added before determining the cell counting at 654 nm in a plate reader. Relative fluorescence units (RFU) were normalized to the cell counting measured by the absorbance.

Real time PCR analysis of mRNA and microRNAs

MDA-MB231 breast cancer cells ($3x10^5$ cells/well) were seeded in 6-well plates and incubated for 24 h to allow cell attachment before treatment with different mango extract concentrations (1.25 – 10 ug/ml) for 24 h. mRNA and miRNA were isolated using the mirVana^{TM} miRNA Isolation Kit (Applied Biosystems, Foster City, CA) following the manufacturer's recommended protocol. The quality and quantity of RNA samples were assessed using the NanoDrop ® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) at 260 and 280 nm. The isolated RNA was used to synthetize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) following the manufacturer's recommended protocol. Each primer tested was designed using Primer Express software (Applied Biosystems, Foster City, CA), homology searched by an NCBI BLAST and the specificity was examined by a dissociation curve analysis. Real time PCR reactions were conducted using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA). TATA binding protein (TBP) was used as mRNA endogenous control. Primers were purchased from Integrated

DNA Technologies, Inc. (San Diego, CA) and the sequences of the primers used were as follows:

TBP F: 5'- TGCACAGGAGCCAAGAGTGAA - 3'

TBP R: 5'- CACATCACAGCTCCCCACCA - 3'

NFKB F: 5'- TGGGAATGGTGAGGTCACTCT - 3'

NFKB R: 5'- TCCTGAACTCCAGCACTCTCTTC - 3'

PARP-1 F: 5'-AGA TGG TGG ACC CGG AGA A – 3'

PARP-1 R: 5'- GAC AAA GCA GCC TGG ATGGT – 3'

SURVIVIN F: 5'- CCATGC AAA GGA AAC CAA CAAT – 3'

SURVIVIN R: 5' – ATG GCA CGG CGC ACT T - 3'

BCL2 F: 5'-GATACGCACCCCCCAACTC-3'

BCL2 R: 5'-CGGAAGTCACCGAAATGTTCA-3'

VEGF F: 5'-AAAGGCCGTGTCATCGTTTC-3'

VEGF R: 5'-CCATATGCGGTACAAGTCAGG-3'

AKT F: 5'-TCCCGAGGCCAAGTCCTT-3'

AKT R: 5'-CCGCCAAGCCTCTGCTT-3'

HIF-1α F: 5' – GTT TAC TAA AGG ACA AGT CAC C – 3'

HIF-1α R: 5' – TTC TGT TTG TTG AAG GGAG – 3'

mTOR F: 5' – CAA ACA GTT CAC CCT CAG T – 3'

mTOR R: 5' – GCT GCC ACT CTC CAA GTT TC – 3'

PI3K p85β F: 5'- CCT GGC ACC TAT GTG GAG TT – 3'

PI3K p85β R: 5'- ACA TCA GGT GGG GAG AAC TG – 3'

PTEN F: 5'-ACCAGGACCAGAGGAAACCT-3'

PTEN R: 5'-GCTAGCCTCTGGATTTGAGC-3'

Quantification and analysis of miRNA-21 and RNU6B (endogenous control) were assessed using the Taqman® MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA) as previously described (*105*). Briefly, the reverse transcription samples were diluted in a 1:15 ratio and amplified with Taqman ® Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems, Foster City, CA).

Transfection assay

Cells ($3x10^5$) were seeded in 6-well plates and transfected with 20nM miRNA-21a (Dharmacon, Lafayette, CO) as previously described (*105*) using Lipofectamine 2000 (Invitrogen, Carlsband, CA) for 6 h. After transfection cells were treated with 10 μg/ml of mango extract for 24 h and collected for RNA extraction.

Protein expression

MDA-MB231 breast cancer cells were grown ($3x10^5$ cells/well) in 6 well plates and allowed to attach for 24 h prior treatment with different mango polyphenol concentrations (1.25 – 10 mg GAE/mL) for 24 h. Cells were harvested and cell lysates were obtained using the Pierce Ripa buffer (25mM Tris®HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS) supplemented with Halt protease and

phosphatase inhibitors (Thermo Scientific, Rockford, IL.). Protein content was assessed using the Bradford reagent (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. Proteins were separated on a 7.5, 10 and 20% SDS-PAGE at 120 V for 1 h and transferred to PVDF membrane (Bio-Rad, Hercules, CA) as previously performed (*105*). Membranes were blocked with 5% non- fat milk in 0.1% Tween-PBS (T-PBS) solution for 1 h and incubated with primary antibodies (1:1000) in 3% bovine serum albumin in T-PBS overnight at 4C with gentle shaking. After 3 washing steps with T-PBS for 5 minutes each, membranes were incubated with secondary antibodies (1:2000) in 5% non-fat milk dissolved in T-PBS for 1 h. Membranes were washed three times for 15 minutes and proteins were visualized with a Kodak Molecular Imaging System (Carestream Health, Rochester, NY).

The Luminex® assay was assessed using an 11-plex AKT/mTOR phosphoprotein magnetic bead kit (Millipore, Billerica, MA) following the manufacturer's protocol. Data were analyzed using Luminex xPonent 3.0 software.

Statistical analysis

Data from *in vitro* experiments were analyzed by one-way-analysis of variance (ANOVA) using SPSS (SAS Institute Inc., Chicago, IL). Data represents mean values with their standard deviations (SD) or standard error (SE), corresponding to three or more replicates. Differences were deemed significant at $p < 0.05$ using a Duncan pairwise comparison. For transfections with antagomir miRNA-21, differences were deemed significant at $p < 0.05$ using a *t*-student comparison

Results and Discussion

Chemical composition of mango Keitt polyphenols

Several studies have reported that mango varieties are a rich source of a diverse class of polyphenols such as gallotanins, gallic acid, galloylglucosides, and flavonoids. In addition, mangiferin and a complex mixture of other polyphenols have also been reported. In this study, the main polyphenol compounds identified in mango Keitt pulp were gallic acid, hydroxybenzoic acid hexoside, and hydrolysable tannins such as mono-galloylglucoside and many others with different degree of galloylation ranging from penta-O-galloylglucoside to nona-O-galloylglucoside (Figure 21, Table 6). Similar results were reported by previous studies (*40, 44, 108*). Mangiferin was not detected in mango Keitt pulp. It is important to mention that mangiferin is a water soluble xanthone which is commonly found in the mango peel, therefore it was not detected since the polyphenol extraction was performed using the mango pulp. In addition, the content of mangiferin varies depending on several factors, including the cultivar. For instance, mangiferin was not detected at all in pulps of some of the 14 mango varieties from Africa, Asia, Australia and South America (*109*). In addition, it has been reported that factors such as the soil, ripening stage, harvest time and storage conditions are considered to affect the polyphenol contents of fruits, leading to a variability of the bioactive compounds (*110*).

The total C_{18} polyphenolic content of mango Keitt variety assessed by the Folin-Ciocalteu assay as well as the antioxidant capacity is shown in Table 5.

Table 5. Total C_{18} polyphenol content and antioxidant activity of mango Keitt extract

Mango Variety	Total C_{18} polyphenolic content (mg of GAE/gr. of pulp)	Antioxidant activity (µmol of TE/gr. of pulp)
Keitt	77.7	19.2

The polyphenolic content found in mango Keitt extract was 77.7 mg of GAE/gr. of pulp. Previous studies have reported a higher polyphenolic content in mango pulp (*40*). It should be noted that the polyphenolic content of mango Keitt extract comes from a C_{18} cartridge which can bind polar molecules such as sugars and vitamin C. The absence of these molecules in the final crude extract may have contributed to low polyphenolic content since any reducing agent such as sugars and vitamin C can react with the Folin-Ciocalteu method. Moreover, some factors are indicated to affect the polyphenol composition such as fruit ripening and method of extraction which in this study may have also affected the polyphenol content in the final crude extract.

Individually, most of mangoes' polyphenols contribute to their total antioxidant capacity. The antioxidant activity of mango Keitt extract was 19.2 µmol of TE/gr. of pulp. Previous studies have reported greater value; however tannins, in general, show high antioxidant potential due to their high molecular weight and high degree of hydroxylation of aromatic rings (*111*). In addition, Hagerman et al. (*112*) suggested that tannins may be much more potent antioxidants than simple monomeric phenolic compounds. Since hydrolysable tannins are one of the major polyphenol compounds

present in mango Keitt pulp, the antioxidant activity may be dependent on the many hydroxyl groups as well as the degree of polymerization; conferring them a better peroxyl radical scavenging capacity which may be presumably shown at even low concentrations.

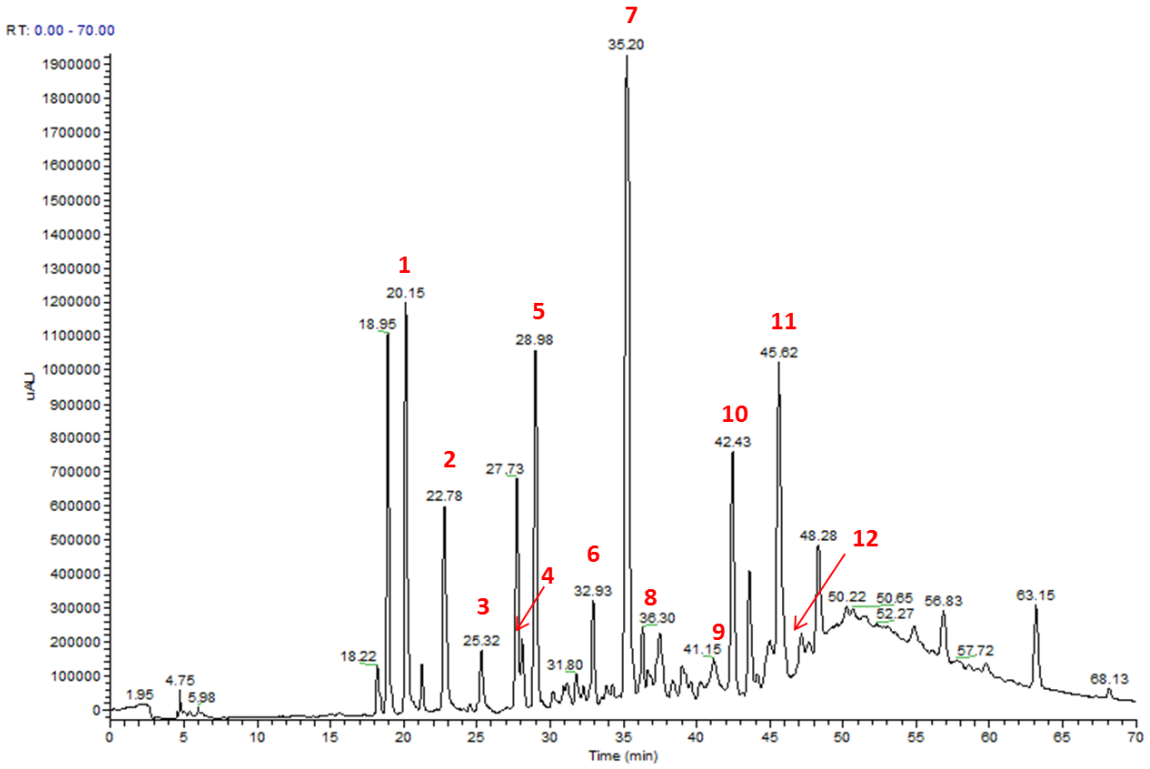

Figure 21. Representative chromatogram of mango Keitt polyphenols at 280 nm

Table 6. Tentative characterization of mango Keitt polyphenol extract

Peak No	RT (min)	λ_{max} (nm)	Compound	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)	Concentration (ppm)
1	20.15	278	Mono galloyl-glucoside	331.1	271.1, 169.2, 211.1	27.29
2	22.78	271	Gallic Acid	169.2	125.2	16.62
3	25.32	270	Mono-Galloyl di-glucoside	493.2	313.10, 271.15, 331.09	3.05
4	28.09	270	Mono galloyl glucoside	331.1	169.2	4.50
5	28.98	260	hydroxybenzoic acid hexoside	299.1	1373.1, 179.0, 239.1, 208.9	52.86
6	32.93	290	Coumaric acid hexoside	324.79	163.12	3.30
7	35.2	266	Dihydrophaseic acid	443.1	237	69.79
8	36.3	314	Coumaric acid hexoside	325	145.3, 187.2, **163.2**, 265.1, 119.3	1.96
9	41.15	252, 379	Sinapic acid hexoside	385.02	223	
10	42.43	266	Dihydrophaseic acid	443.18	425.35, 237.13, 130.8	19.92
11	44.92	278	Penta-galloyl glucoside	939.14	769.10, 787.1, 617.14	29.54
12	47.15	278	Hexa-galloyl glucoside	1090.95	939	1.98

Effects of mango Keitt polyphenol extract (ME) on MDA-MB231 breast cancer cell growth

Uncontrolled cell proliferation contributes, among other factors, for cancer evolution and progression (*144*). Therefore, the anti-proliferative effects of mango Keitt polyphenols were evaluated in MDA-MB231 breast cancer cells. The net growth of breast cancer cells was performed by cell counting after mango polyphenol treatment (1.25 – 10 µg GAE/ml) for 48 h. Results showed a significant decrease in cell growth in a dose dependent manner when ME was applied to MDA-MB231 breast cancer cells (Figure 22).

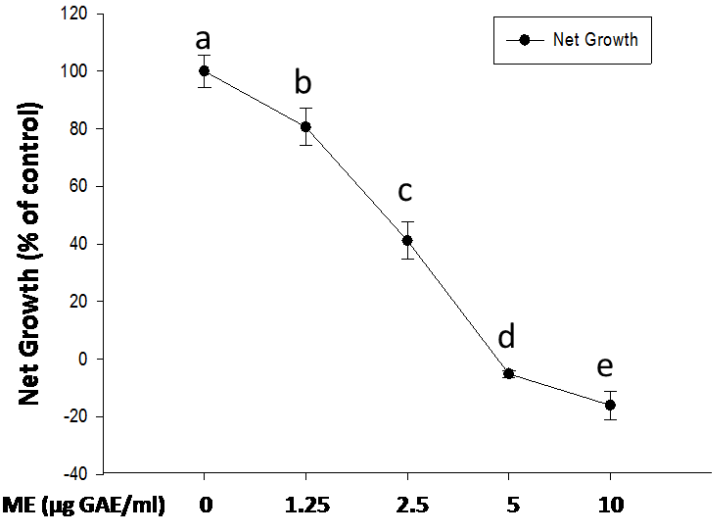

Figure 22. Cell proliferation of MDA-MB231 breast cancer cells treated with ME. Cells were treated with different concentrations of ME and cell growth was assessed after 48hr of incubation. Values are means ± SE (n=3). Different letters indicate significance at p <0.05

These results are consistent with previous reports, where mango polyphenols (1.25 – 50 mg GAE/L) extracted from the edible portion of Ataulfo and Haden mango

varieties showed cell growth suppression in a dose dependent manner when tested in several cancer cell lines including MDA-MB231 breast cancer cells (*40*). Pomegranate is a rich tannin-containing fruit which has shown cytotoxic effects in MDA-MB231 and BT-474 breast cancer cell lines *in vitro* and *in vivo* (*63*). In contrast, another study reported the low cytotoxic efficacy of mango Ataulfo polyphenols after a 72 h treatment in MCF-7 breast cancer cell line; however mango polyphenols were extracted in water and did not contain higher molecular weight gallotannins which are reported in this study. Gallotannins are considered as major antioxidant polyphenols found in mango fruit (*41*), thus the degree of galloylation may have a potential effect in the anticancer activities exerted by mango polyphenols (*40, 145*). Mango is also a fruit rich in phenolic acids, which have been shown to exert anti-proliferative action. A study reported that a group of selective phenolic acids exerted anti-proliferative and apoptotic effects in breast cancer cells at low concentrations (*146*).

As a comparison to the results shown in Figure 22, it is important to mention that ME suppressed the growth of breast cancer cells at 5 μg GAE/ml, whereas at the same concentration the growth of MCF-12A non-cancer cells was inhibited only by ~20%. This results are indicating that mango polyphenols may have a potential role in chemoprevention since bioactive natural compounds which have growth suppressive effects in cancer cells but do not interfere with the growth of non-cancer cells might be considered as chemopreventive agents (*40*).

Effects of mango Keitt polyphenols extract (ME) on the generation of reactive oxygen species

Reactive oxygen species can cause DNA damage which has been indicated as one of the major causes of cancer (*22, 113, 147*). In general, higher levels of ROS are produced by cancer cells at a faster rate than non-cancer cells due to an increased metabolism and mitochondrial activity, among other factors (*148*). The oxidative DNA damage induced by ROS may be involved in the development of breast cancer (*149*). In fact, increased levels of DNA damage caused by ·OH attack have been reported in inflammatory breast disease (*113, 149*). In addition, greater oxygen-free-radical production may enhance oxidative stress within breast carcinogenesis (*150*).

The generation of reactive oxygen species was evaluated in MDA-MB231 breast cancer cells after a 24 h. treatment with ME (1.25-10 μg GAE/ml). In this case, TNF-α was not used to promote inflammation or ROS levels since cancer cells are usually under oxidative stress (*148*). Treatment of MDA-MB231 breast cancer cells with ME for 24 h. significantly reduced ROS levels by ~ 25% compared to the control; however no significant differences were observed between the different concentrations of ME evaluated (Figure 23). A similar trend was found in MCF-12A non-cancer breast cells in which ME significantly reduced ROS generation after 24 h. treatment but no significant changes between the concentrations of ME were found (Figure 11). These results are indicating that the ability of mango Keitt polyphenols to quench or trap free radicals is not dependent on the concentration and/or the cell line tested.

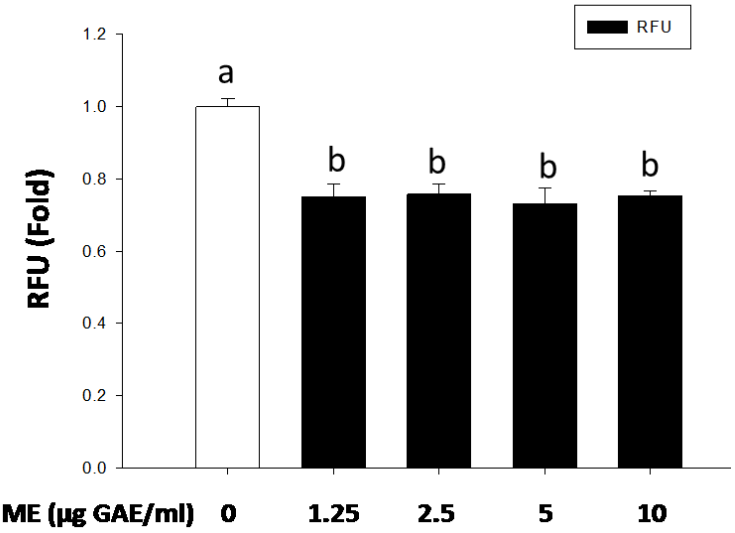

Figure 23. Generation of reactive oxygen species in MDA-MB231 breast cancer cells treated with ME. ROS generation was detected using 2,7 dichlorofluorescein diacetate (DCFH-DA). The fluorescence signal was monitored as described in Materials and Methods. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$.

Several studies have previously demonstrated how polyphenols may target the production of ROS in both cancer and non-cancer cells. A study reported that polyphenols extracted from mango Ataulfo reduced ROS levels by up 17-72% in colon cancer cells. The same study reported a greater inhibition of ROS production in non-cancer colon cells (*40*) Another study showed that mango polyphenols extracted from the kernel scavenged superoxide anions by applying an electron spin resonance spin trapping method (*151*). In general, individual polyphenols present in mango such as hydrolysable tannins have shown to possess great antioxidant activity. In fact, hydrolysable tannins along with condensed tannins have shown better peroxyl radical quenching capacity than simple phenols due to the presence of many hydroxyl groups

and degree of polymerization (*112, 118, 119*). In addition, a study demonstrated the potent radical scavenging effects of penta-O-galloylglucoside derived from mango peel on hydroxyl radical (OH) superoxide anions (O_2^-) and singlet oxygen (O_2) production. The authors suggested that the great antioxidant activity of penta-O-galloylglucoside was partly due to the number of galloyl groups in the molecule which in turn may increase its scavenging effects. The findings in this study are suggesting that hydrolyzable tannins such as penta-O-galloylglucose may be partly responsible for the antioxidant activity exerted by ME in both cancer and non-cancer breast cells. Overall, the ability of polyphenols present in ME to scavenge free radicals and to mitigate a persistent oxidative stress in breast cancer cells was demonstrated suggesting the great antioxidant capacity of mango polyphenols.

ME decreases inflammation and angiogenesis in MDA-MB231 breast cancer cells

Inflammation can accelerate the carcinogenesis development by different mechanisms. It enhances the proliferation and survival of malignant cancer cells, promotes angiogenesis and metastasis, subverts adaptive immune responses, and alters responses to hormones and chemotherapeutic agents (*5*). Therefore, the anti-inflammatory properties of ME were assessed in MDA-M231 breast cancer cells after 24 h treatment. ME down-regulated the expression of inflammatory markers such as NF-κB subunit p65 and TNF-α in a dose response manner at the mRNA level (Figure 24, A, B). At 10 μg GAE/ml, the expression of NF-κB and TNF-α was decreased by 0.35 and 0.10 fold compared to the control, respectively. The expression of NF-κB (p65) and its

phosphorylated form was also decreased at the protein level as shown in Figure 25. This was accompanied by a decreased expression of the angiogenic marker, vascular endothelial growth factor (VEGF). Results are showing that ME down-regulated the expression of VEGF mRNA to 0.01 fold compared to the control (Figure 24, C). Moreover, the protein expression of VEGF was also decreased by ME treatment as shown in Figure 25.

Increased circulating levels of TNF-α in breast cancer has been linked to an up-regulation of a number of genes involved with proliferation, invasion and metastasis (*4*) such as NF-κB, which is known to play a critical role in cancer and various chronic diseases. Therefore the inhibition of NF-κB is an attractive target for therapeutic development (*152*). In this study, ME decreased both TNF-α and NF-κB mRNA expression in a dose dependent manner suggesting the potential anti-inflammatory properties that mango Keitt polyphenols may exert in a highly metastatic breast cancer cell line such as MDA-MB231. The down-regulation of NF-κB by ME was accompanied by a significant decreased expression of vascular endothelial growth factor (VEGF) which is known to play an important role in angiogenesis. VEGF is commonly expressed in breast cancer cells and tumors and the inhibition of its activity could be a new approach for the treatment of breast cancer by preventing angiogenesis (*153*). In this study, ME decreased VEGF mRNA and protein expression suggesting the potential angiogenic properties of ME.

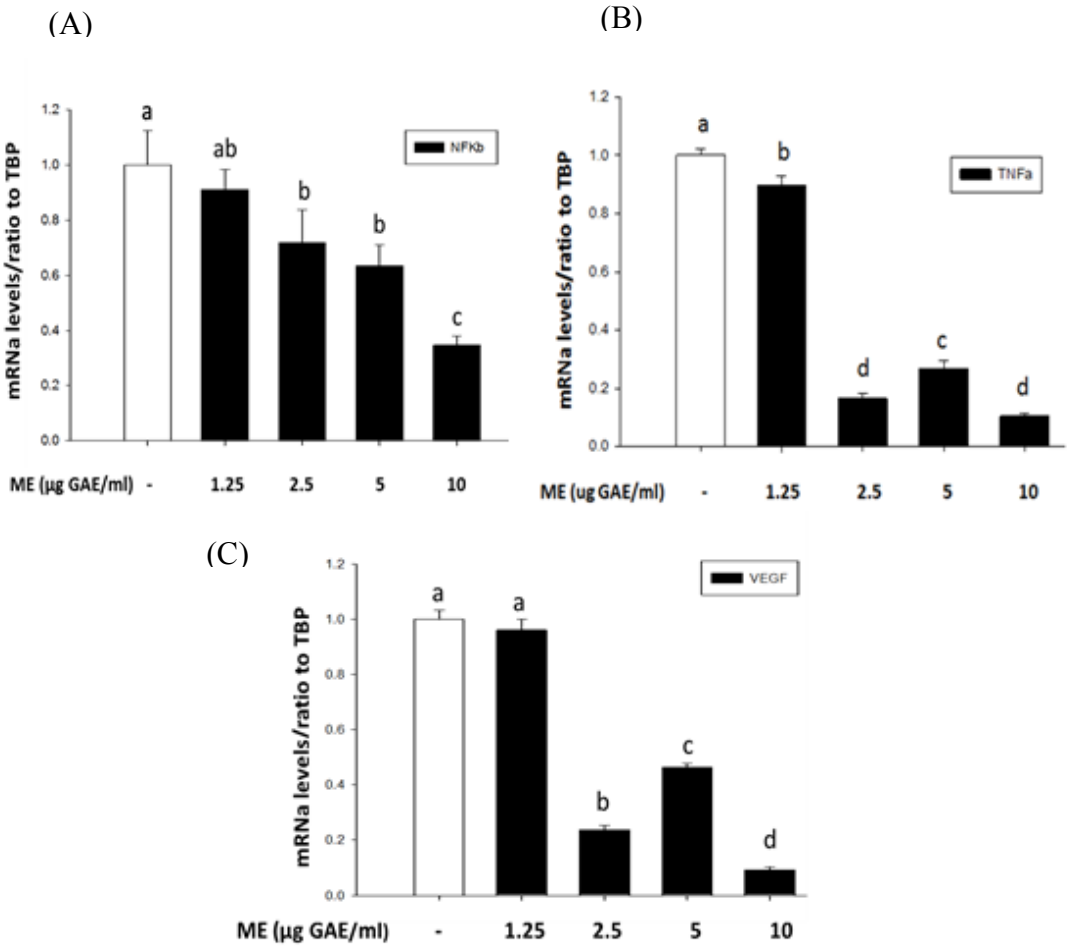

Figure 24. Gene expression of inflammatory (A) NF-KB, (B) TNF-α and angiogenic (C) markers in MDA-MB231 breast cancer cells after 24 h. treatment wih ME. Gene expression was analyzed by qRT-PCR as a ratio to TATA binding protein (TBP) mRNA. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$.

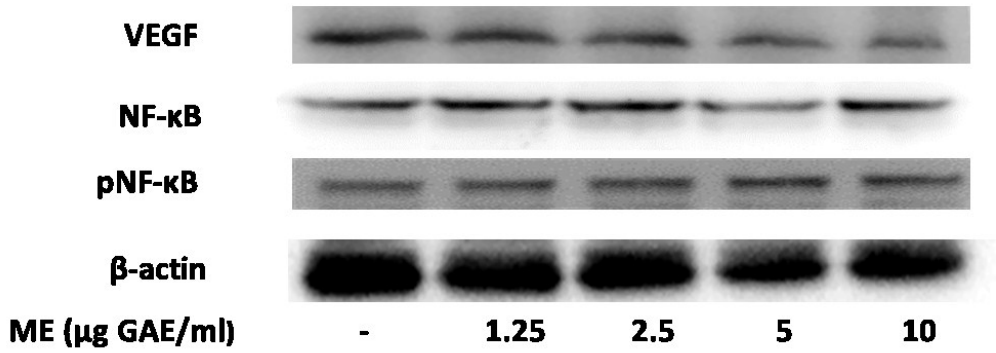

Figure 25. Protein expression of angiogenic and inflammatory genes. MDA-MB231 breast cancer cells were treated with different concentrations of ME for 24 hr. and the expression of VEGF, NF-κB and pNF-κB proteins on whole cell lysates was determined by Western Blot as described in Materials and Methods.

Overall, these results are consistent with previous reports in which bioactive compounds decreased the expression of NF-κB and related inflammatory genes (*154-156*). As a comparison to another fruit rich in tannins, a pomegranate extract (2.5-10 μg/ml) down-regulated the expression of NF-κB and VEGF in MDA-MB231 and BT-474 breast cancer cells (*63*). Previous studies reported that gallic acid was able to exert anti-carcinogenic effects in several *in vitro* and in *vivo* models. Moreover, gallic acid has been indicated as an essential anti-cancer component in a standardized extract derived from mango bark (Vimang) by inhibiting NF-κB activity in the cancer-inflammation network in MDA-MB231 breast cancer cells (*157*). In addition, the potential mechanism of hydrolyzable tannins such as penta-O-galloylglucose include anti-angiogenesis and anti-proliferative actions through inhibition of DNA as well as induction of apoptosis and anti-inflammatory properties in prostate, breast, lung and other cancer cell lines (*47*).

In this study, gallic acid, hydrolysable tannins and other polyphenols in mango Keitt are likely to be responsible for the anti-inflammatory and anti-angiogenic properties exerted in MDA-MB231 breast cancer cells. In addition, ME polyphenols may be considered as a novel preventive strategy against breast cancer since it can modulate the expression of NF-κB and related downstream effectors.

Modulation of apoptotic markers by ME

Cancer cells are characterized by a deregulated proliferation and/or inability to undergo apoptosis (*26*). Therefore induction of apoptosis is considered to be one of defensive mechanisms against cancer development (*158*). The expression of apoptotic markers in MDA-MB231 was evaluated after 24 h. treatment with ME. Results are showing a concentration-dependent decrease in the BCl2 and Survivin gene expression by 0.05, 0.37-fold respectively compared to the control when the highest concentration of ME (10 μg GAE/ml) was applied to MDA-MB231 breast cancer cells. PARP-1 gene expression was also down-regulated by ME treatment to 0.11-fold compared to the control (Figure 26). Moreover, at the protein level, the activated form of caspase-3, a major apoptosis executing enzyme, was induced by ME at 2.5 and 5 ug GAE/ml. This was accompanied by an increased expression of cleaved Poly (ADP-ribose) polymerase 1 (PARP), which is a substrate for caspase-3. Full lengths of cleaved PARP-1 protein were increased by ME in a dose response manner. Moreover, the activation of cleaved caspase-3 by ME treatment was accompanied by an increased expression of cytochrome C, whereas the BCl2 protein expression was down-regulated by ME. (Figure 27).

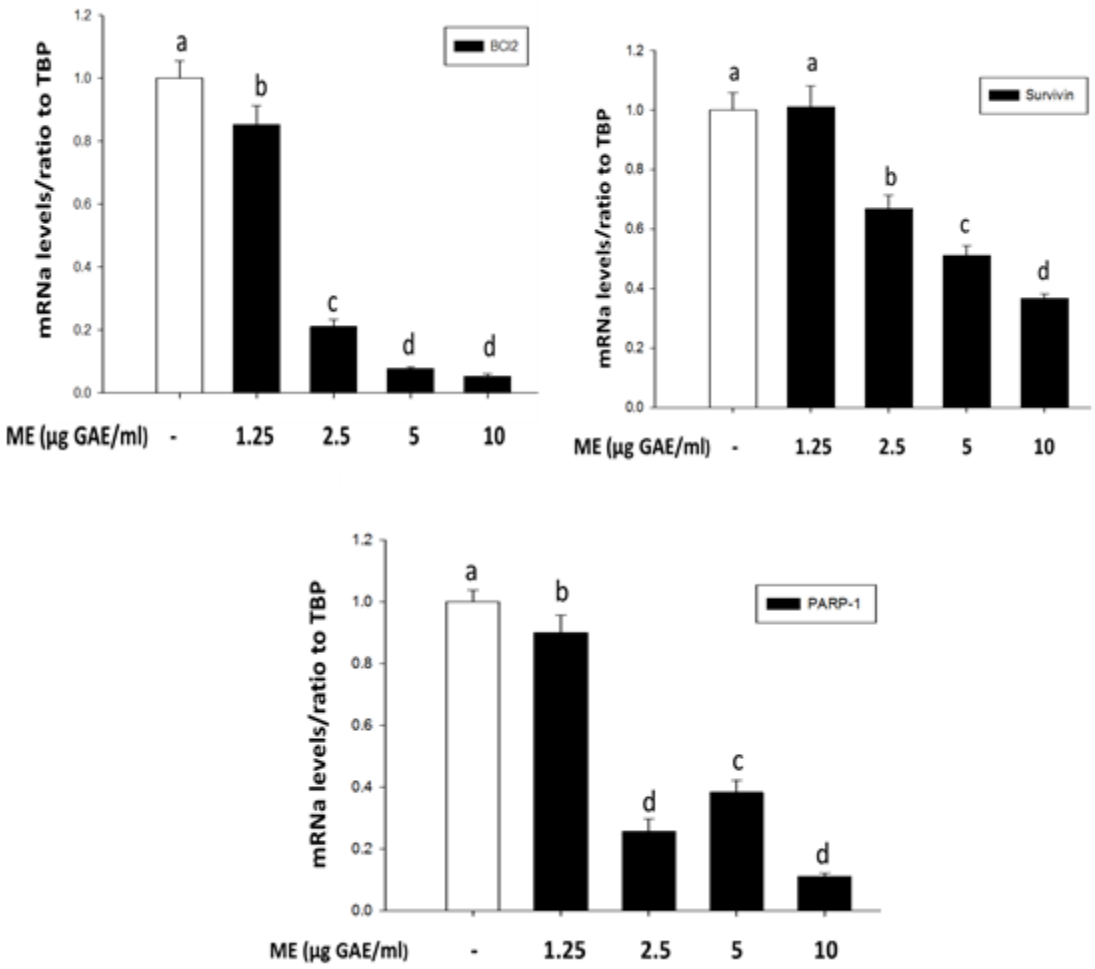

Figure 26. Gene expression of apoptotic markers BCl2, Survivin and PARP-1 in MDA-MB231 breast cancer cells after 24 h. treatment with ME. Gene expression was analyzed by qRT-PCR as a ratio to TATA binding protein (TBP) mRNA. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$

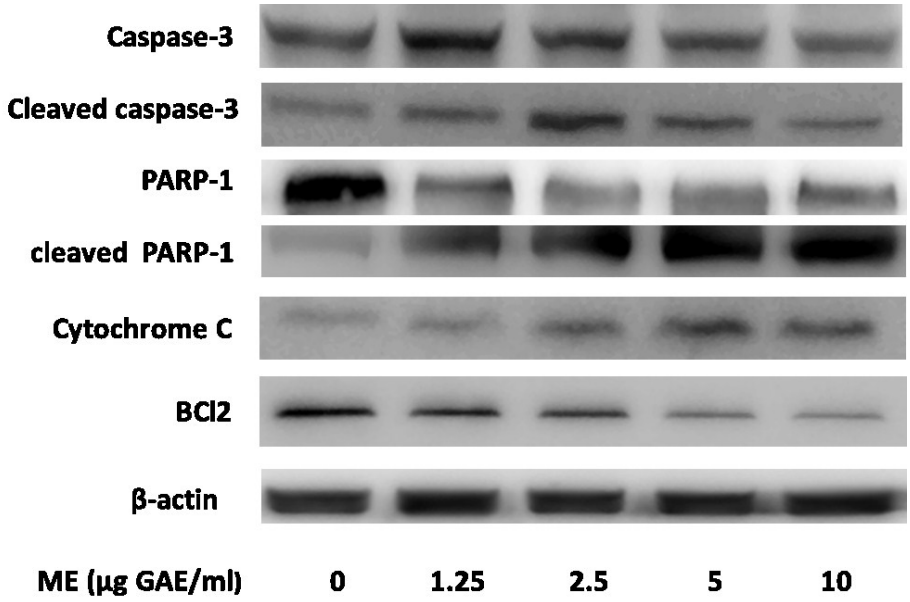

Figure 27. Protein expression of apoptotic markers. MDA-MB231 breast cancer cells were treated with different concentrations of ME for 24 hr. and the expression of caspase-3, cleaves caspase-3, PARP-1, cleaved PARP-1, cytochrome C and BCl2 on whole cell lysates was determined by Western Blot as described in Materials and Methods.

The cytotoxic effects exerted by mango Keitt polyphenols (Figure 27) reflected an increased expression of cleaved caspase-3, which is the activated form of caspase-3, a major executive enzyme used as a commonly indicator for the induction of apoptosis (*26*). PARP-1 is an enzyme known for maintaining genome integrity and is cleaved during apoptosis by caspase-3 (*159*). Polyphenols have previously shown to induce apoptosis in cancer cell lines (*160-162*) involving caspase-3. The results in this study are in accordance with previous findings which reported the induction of apoptosis exerted by polyphenol compounds in colon and breast cancer cell lines through the involvement of caspase-3 and subsequent PARP-1 cleavage (*63, 159*). Survivin is involved in the inhibition of apoptosis and exerts multiple effects throughout the cell cycle (*158*). The

results in this study showed that ME treatment reduced the gene expression of survivin, which may have contributed to induce apoptosis in MDA-MB231 breast cancer cells. In addition, higher doses of ME (5-10 ug GAE/ml) resulted in a decreased gene and protein expression of BCl-2, an anti-apoptotic protein which controls main steps in cell death (*26*) and prevents cells from undergoing apoptosis (*163*). Previous studies have reported the apoptotic activity of mango polyphenols in cancer cell lines. For instance, an ethanolic extract of mango peel (Ataulfo cv.) induced apoptosis in human cervical cells (HeLa) by down-regulating the anti-apoptotic expression of BCl-2, resulting in the activation of caspase-3 and the degradation of PARP-1 (*164*). Another study showed that a mango (Fozli cv.) peel extract down-regulated the expression of BCl-2 and triggered the proteolytic activation of caspases-3, -8, and -9 and the degradation of poly ADP-ribose polymerase in HeLa cells (*99*).

The overexpression of BCl-2 blocks the cytochrome C release from the mitochondria preventing the initiation of the apoptotic program (*163*). ME showed the capacity to inhibit the gene and protein expression of BCl-2 and as a consequence, the release of cytochrome C from the mitochondria into the cytosol was fostered in a dose response manner after ME treatment for 24 h. (Figure 27). These results are indicating the ability of ME polyphenols to enhance the apoptotic program in breast cancer cells by the mitochondrial or intrinsic pathway.

The apoptotic activities of isolated polyphenols compounds present in ME have been previously reported. A gallotanin-rich *Caesalpinia spinosa* fraction induced apoptosis with mitchocondrial potential loss, caspase-3 activation and DNA

fragmentation in the murine mammary tumor cell line 4T1 and leukemia K562 cells (*165, 166*). In addition, gallic acid has demonstrated its ability to regulate pro and anti-apoptotic genes in leukemia (*167, 168*) and lung cancer (*169*) cell lines. Overall, the regulation of apoptosis exerted by ME in MDA-MB231 breast cancer cells may be partly due to the synergistic activities of the single polyphenol compounds present in mango Keitt pulp.

ME modulates the PI3K/AKT/mTOR pathway in MDA-MB231 breast cancer cells and increases the expression of phosphatase and tensin homolog (PTEN)

The phosphatidylinositol 3-kinase (PI3K) signaling axis influences on cancer cell growth, survival, motility, and metabolism (*170*) This signaling pathway is activated and deregulated in a wide variety of cancers (*171*) and inhibitors of this pathway are under active development as potential anticancer therapeutics (*73, 76, 77, 131, 170, 171*). In this study, ME down-regulated the expression of PI3K accompanied by a decreased mRNA expression of AKT, mTOR and HIF-1 α (Figure 28). After 24 h. treatment with ME, the gene expression of PI3K, AKT, mTOR and HIF-1α was decreased to 0.03, 0.22, 0.55 and 0.28 fold, respectively, compared to the control when MDA-MB231 breast cancer cells were treated with the highest concentration of ME (10 μg GAE/ml). This was accompanied by a decreased protein expression of PI3K, AKT and mTOR in a dose dependent manner as shown in Figure 29. Phosphatase and tensin homolog (PTEN) is a phosphatase that degrades the phosphoinositide products of PI3K and acts as a tumor suppressor in a large number of cancers, including breast cancer

(*131*) ME up-regulated the mRNA expression of PTEN up to 1.9-fold when higher concentrations of ME (5-10 μg GAE/ml) were applied to MDA-MB231 breast cancer cells. Similar results were observed when the protein expression of PTEN and its phosphorylated form were evaluated since their expression was regulated in a dose-response manner after ME treatment for 24 h (Figure 30 A,B).

The PI3K/AKT signaling pathway is currently considered as an attractive target for effective therapeutic strategies (*73, 131, 171, 172*). In this study, ME treatment showed a significant decrease in the PI3K mRNA expression; however, this inhibition was not in a dose response manner as at 5 μg GAE/ml there was a slight increase in the expression of PI3K compared to 2.5 and 10 μg GAE/ml of ME. In contrast, full lengths of PI3K protein were decreased by ME in a dose response manner. As far as AKT, which is the major downstream target of PI3K, results are showing a concentration-dependent decrease in both mRNA and protein expression. The PI3K/AKT has an influence on cell survival pathways through the phosphorylation of downstream genes such as mTOR and NF-κB (*103*) . Results in this study are showing a decrease in the mTOR mRNA expression compared to the control; however, there were no significant changes between the different doses of ME applied to MDA-MB231 breast cancer cells. In contrast, ME treatment exerted a concentration-dependent decrease in the mTOR protein expression as shown in Figure 29.

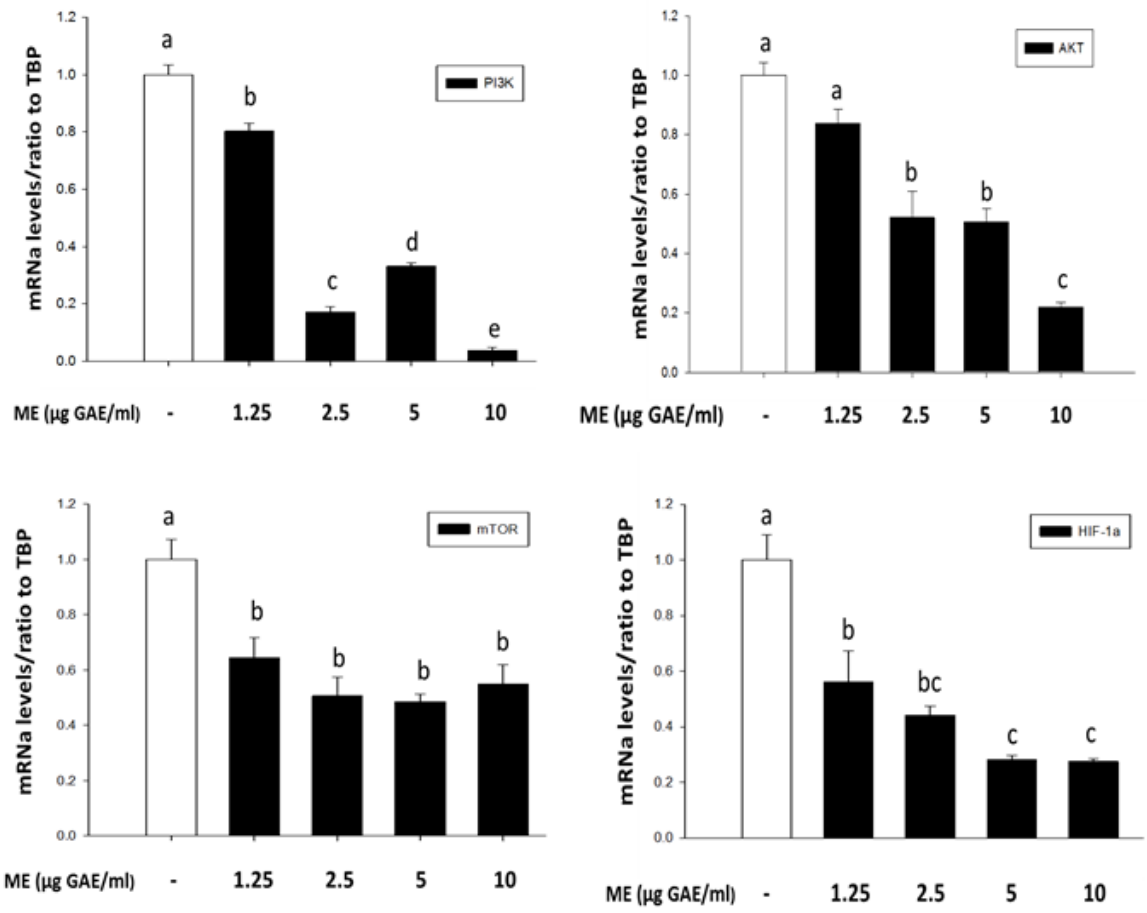

Figure 28. Gene expression of PI3K, AKT, mTOR and HIF-1α in MDA-MB231 breast cancer cells after 24 h. treatment with ME. Gene expression was analyzed by qRT-PCR as a ratio to TATA binding protein (TBP) mRNA. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$

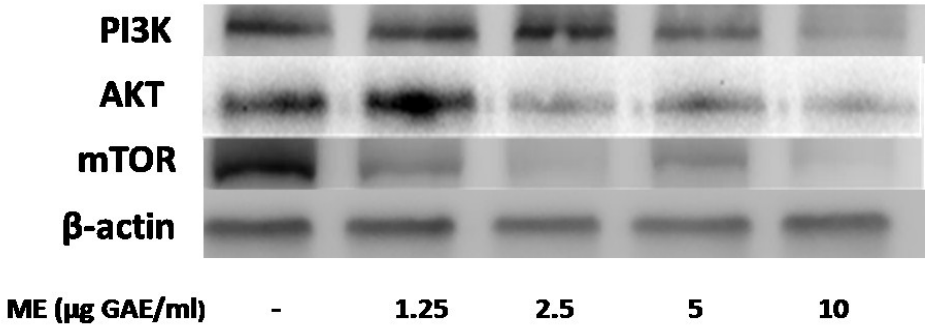

Figure 29. Protein expression of PI3K, AKT and mTOR in MDA-MB31 breast cancer cells treated with different concentrations of ME for 24 h. as determined by Western Blot analysis.

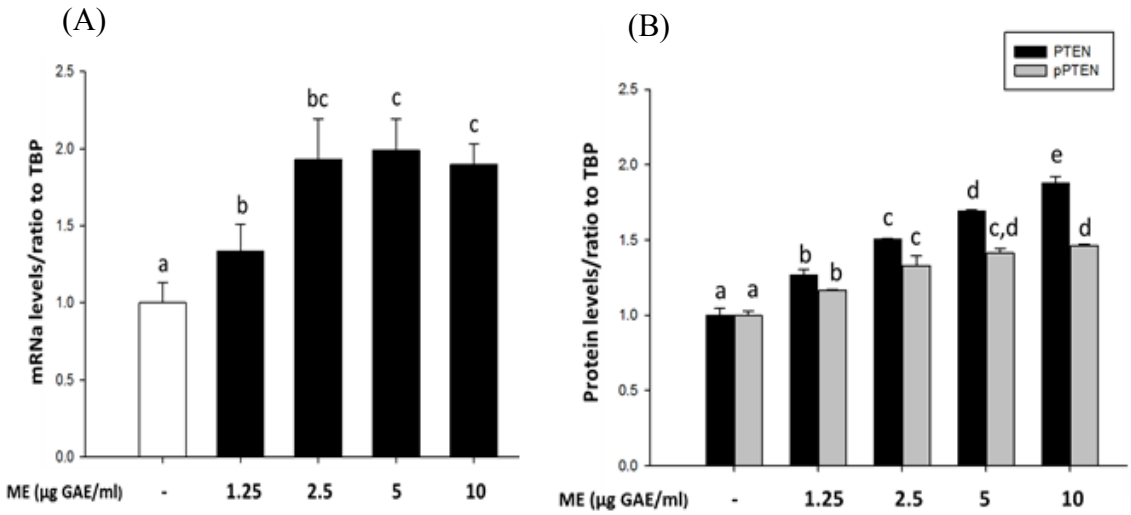

Figure 30. (A) PTEN mRNA expression after 24 h. treatment with different concentrations of ME. Gene expression was analyzed by qRT-PCR as a ratio to TATA binding protein. (B) PTEN and pPTEN protein expression after 24 h. treatment with different concentrations of ME. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$

The hypoxia-inducible factor alpha(HIF-1α) is a downstream target of mTOR in the PI3K/AKT pathway (*84*). In addition, inhibition of PI3K results in a decreased HIF-1α activation (*81*). Results in this study are showing that HIF-1α mRNA expression was decreased up to 0.45 fold compared to the control even when low concentrations of ME (1.25 and 5 ug GAE/ml). At higher concentrations of ME (5 and 10 ug GAE/ml), HIF-1α mRNA expression was inhibited up to 0.2 fold compared to the control (Figure 28). It is well establish that VEGF is a downstream gene of HIF-1α, thus the inhibition of HIF-1/VEGF is associated with a decreased angiogenesis process (*82*). In this study, the inhibition of HIF-1α was accompanied by a decreased expression of VEGF as previously shown indicating that ME polyphenols may be used as an angiogenic inhibitor in the treatment of breast cancer. Additionally, findings in this study are indicating that ME may have a potential and significant impact in cancer cell proliferation and survival by targeting the PI3K/AKT pathway and related downstream targets such as mTOR.

Previous studies have reported the role of several polyphenols such as resveratrol, curcumin, epigallocatechin gallate, indol-3-carbinol, genistein among other compounds, to counteract the expression of PI3K/AKT signaling pathway (*85, 87, 88, 173*). In addition, recent studies reported the effects of tannin-rich fruits on the PI3K/AKT/mTOR pathway. A study reported that a pomegranate extract (2.5-10 μg GAE/ml) inhibited the phosphorylation of PI3K and AKT in MDA-MB231 and BT-474 breast cancer cells suggesting the cytotoxic and anti-inflammatory activities of pomegranate in breast cancer (*63*). A similar study showed that a pomegranate fruit

extract repressed lung tumors in mice by inhibiting the expression of NF-kB, PI3K/AKT and mTOR signaling pathway (*174*).

Individual polyphenols present in mango have been reported to suppress the PI3K/AKT pathway in different *in vitro* and *in vivo* models: i.e., the anti-migratory effect of gallic acid may involve the inhibition of NF-κB activity and multiple proteins related to metastasis and signal pathways, including PI3K in gastric carcinoma (*129*). The anti-inflammatory and anti-carcinogenic activity exerted by mango Keitt polyphenols is attributed, in part, due to presence of high molecular weight such as penta-galloyl-glucose which have shown multiple biological activities *in vitro* and *in vivo* (*47*). In addition, another study showed that penta-galloyl-glucose might inhibit the PI3K/AKT pathway by directly inhibiting the AKT kinase activity in estrogen dependent breast cancer cell lines (*136*). In general, these findings are indicating the potential use of a tannin-rich fruit, like mango as a chemopreventive agent by suppressing the PI3K/AKT/mTOR pathway as well as its downstream effectors.

Effects of ME on the expression of miRNA-21

Small non-coding RNAs or microRNAS (miRNAs) control the expression of hundreds of different genes thus, they are involved in multiple cellular pathways and biological processes (*175*). miRNAs can function as either tumor suppressors or oncogenes. Previous studies have shown that miRNA-21 is a key oncomir overexpressed in a wide of cancers, including breast cancer. This miRNA targets several mRNAs such as PTEN which expression is lost in various cancers (*80*). To investigate the underlying

mechanisms of the up-regulation of PTEN and the suppression of the PI3K/AKT/mTOR pathway, the potential role of miRNA-21 involved in cancer growth and proliferation was investigated. Results are showing that ME polyphenols decreased the gene expression of miRNA-21 in a concentration-dependent manner, thus at 10 μg GAE/ml the expression of miRNA-21 was decreased up to 0.06-fold compared to the control (Figure 31, A). This is the first study indicating the potential role of mango Keitt polyphenols in the down-regulation of miRNA-21. Findings in this study are indicating that polyphenols from mango Keitt could be considered as a novel cytotoxic agent because of its capacity to attenuate the gene expression of endogenous miRNA-21.

In order to demonstrate the involvement of miRNA-21 as an underlying mechanism of the anti-carcinogenic effects of ME polyphenols, MDA-MB231 breast cancer cells were transfected with a specific antagomir for miRNA-21 (ant-miRNA-21). Results showed that miRNA-21 inhibitor decreased the expression of miRNA-21 up to 0.40-fold whereas ME partially reverted the effects of the inhibitor (Figure 31, B). To confirm the involvement of miRNA-21 in the regulation of the tumor suppressor PTEN in breast cancer cells, an antagomir for miRNA-21 was used. Results are showing that the antagomir increased the expression PTEN whereas ME (5 μg GAE/ml) contributed to a more significant expression of this gene (Figure 31, C).

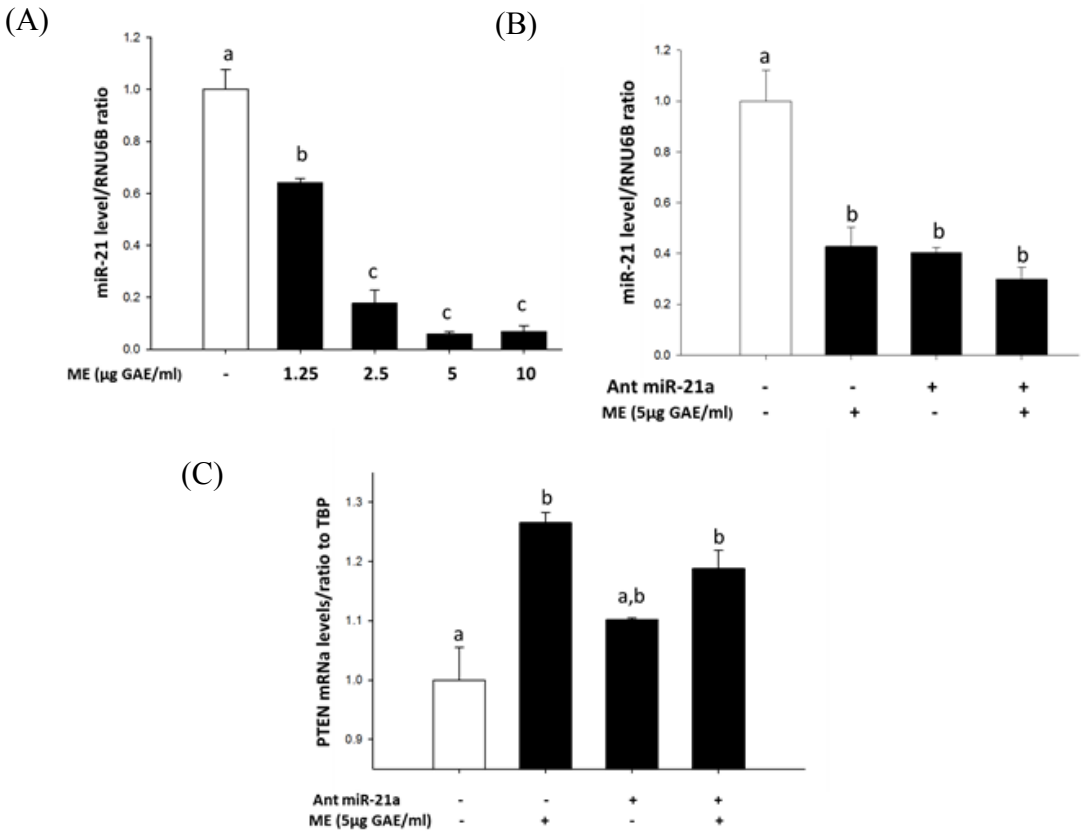

Figure 31. (A) Effects of ME on miRNA-21 gene expression. (B) Effects of ME with and without a specific antagomir (Ant.) for miRNA-21 on the expression of miRNA-21. (C) Effects of ME with and without miRNA-21 antagomir (Ant.) on the gene expression of PTEN. Each experiment was performed at least three times and results are expressed as means ± SE. Different letters indicate significance at $p < 0.05$

Recent data has indicated the relationship between miRNA-21 knockdown and its oncogenic activity. A study reported that the knockdown of miRNA-21 in MCF-7 breast cancer cells led to the suppression of cell growth in a xenograft model *in vivo* (*95, 176*). Another study concluded that the knockdown of miRNA-21 in MDA-MB231 breast cancer cells significantly reduced invasion and lung metastasis (*95, 177*). In addition, Yan et al. (*90*) reported that knockdown of miRNA-21 in MCF-7 and MDA-

MB231 breast cancer cells inhibits *in vitro* and *in vivo* tumor growth. In this study, mango Keitt polyphenols (ME) contributed partially to decrease the expression of miRNA-21 when breast cancer cells were treated with the specific antagomir for miRNA-21. In addition, ME led to an overexpression of the tumor suppressor PTEN even when MDA-MB231 breast cancer cells were treated with the antagomir for miRNA-21 indicating that ME polyphenol treatment may be considered as an inhibitory approach against miRNA-21.

Conclusion

In conclusion, mango Keitt polyphenols exerted cytotoxic effects on MDA-MB231 breast cancer cells by suppressing the generation of reactive oxygen species and modulating of the mRNA and protein expression of apoptotic biomarkers. The anti-inflammatory and anti-proliferative effects of mango Keitt polyphenols were mediated by suppressing the PI3K/AKT/mTOR pathway and relevant downstream effectors such as NF-κB, HIF-1α and VEGF. In addition, the miRNA-21-PTEN/AKT axis was identified as a potential anti-carcinogenic underlying mechanism. Based on these results, mango polyphenols may hold a promising approach in the prevention of breast cancer.

CHAPTER V

OVERALL CONCLUSIONS

Extensive research has been conducted to determine the potential chemopreventive effects of dietary bioactive compounds. So far, the results are promising indicating that dietary polyphenols would be considered as new therapeutic alternatives in the prevention and treatment of chronic-degenerative diseases in a not distant future. However, the need to identify novel and natural sources of dietary polyphenols that could be potentially used in the treatment of breast cancer is increasing.

The general scope of this study was to evaluate the anti-inflammatory and anti-proliferative effects of mango variety Keitt polyphenols in non-cancer MCF-12A and cancer MDA-MB231 breast cells. Results showed that in an inflammatory microenvironment, mango polyphenols were able to counteract the expression of NF-κB which is considered a master regulator of inflammation. In addition, mango polyphenols showed the capacity to suppress the activation of the PI3K/AKT/mTOR pathway as well as to modulate the gene expression of miRNA-126.

Mango polyphenols showed great anti-carcinogenic effects in the highly aggressive MDA-MB231 breast cancer cells by suppressing the generation of reactive oxygen species and inducing apoptotic genes involved in the mitochondrial pathway. The suppression of the PI3K/AKT/mTOR signaling cascade was identified as a potential underlying mechanisms of action by which mango Keitt polyphenols exerted anti-proliferative effects in MDA-MB231 breast cancer cells. These results were

accompanied by a decreased expression of the oncomir miRNA-21 as well as an overexpression of the tumor suppressor PTEN after mango Keitt polyphenol treatment, therefore the miRNA-21-PTEN axis was also linked to the anti-carcinogenic effects of mango polyphenols.

In summary, this *in vitro* study showed that mango variety Keitt is an excellent source of dietary polyphenols with great anti-inflammatory and anti-carcinogenic properties. Findings in this study will provide reference for future *in vivo* experiments as well as clinical applications.

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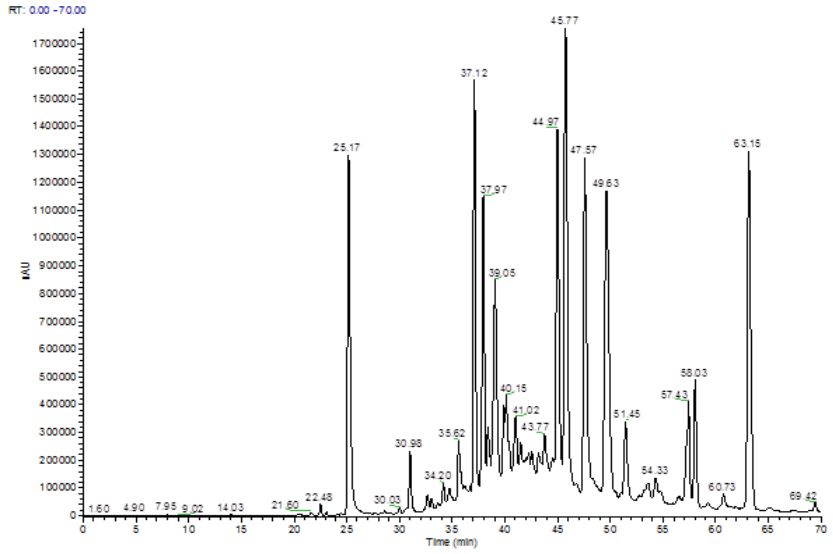
APPENDIX A

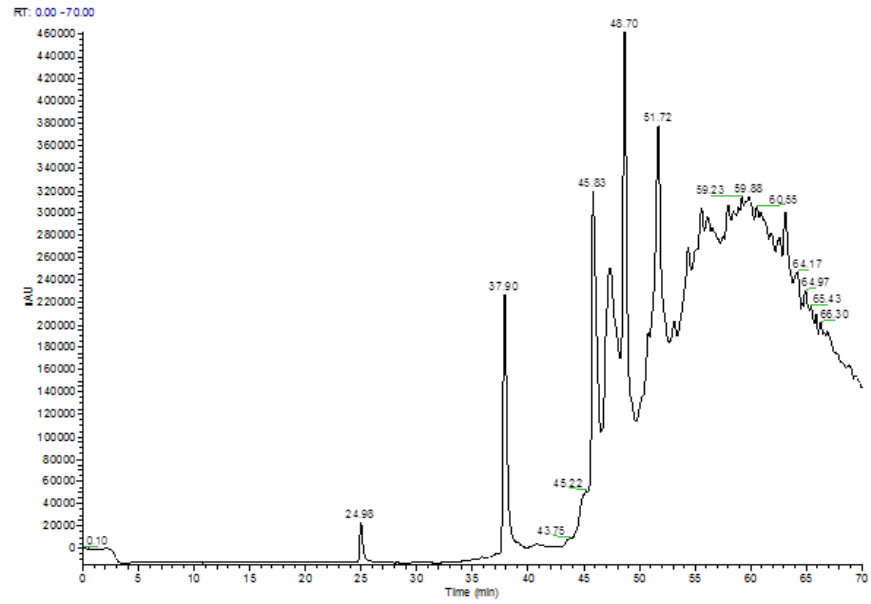
Fractionation of Mango Extract A

Mango Extract A was fractionated onto C18 in high molecular weight fraction (HMW) and low molecular weight fraction (LMW). Briefly, the total phenolic extract was bound onto C18 cartridge, previously conditioned with acidified water and methanol and consecutively eluted with 25% and 100% methanol (v/v). The methanolic fraction (HMW) was evaporated under reduced pressure and the dried fraction was reconstituted in DMSO. The 25% MeOH fraction was evaporated and the aqueous extract was reeluted on C18 cartridge. Polyphenols were washed with 100% methanol and the solvents were evaporated under reduced pressure. The dried extract (LMW) was reconstituted in DMSO. The total C18 polyphenolic content of TPE, LMW and HMW was assessed by the Folin- Ciocalteu assay. The phenolic content of TPE was 26.6 mg GAE/gr. of pulp, whereas the phenolic content of LMW and HMW was 4.7 and 16.7 mg GAE/gr. of pulp, respectively. The HMW fraction presented a phenolic content 3.54 fold-greater than LMW, even when fractions were analyzed at the same dilution (Table 3). The HMW fraction comprised hydrolysable tannins with molecular weights ranging from 787 Da (tetragalloylglucose) to 1547 Da (nonagalloylglucose), while the LMW fraction contained mainly phenolic acids such as gallic acid and hydroxybenzoic acid. The difference in chemical composition may have contributed to a higher phenolic content in the HMW fraction. In fact, tannins have more hydroxyl groups which may lead to an increased reducing capacity shown by the HMW fraction.

(A)



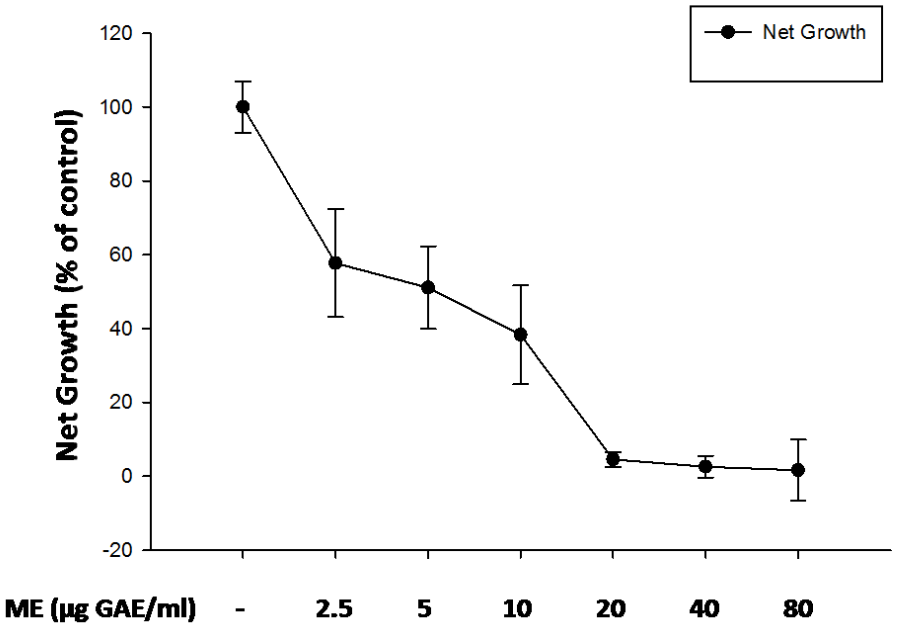
(B)



Representative chromatograms at 280 nm of (A) low molecular weight (LMW) and (B) high molecular (HMW) polyphenolic compounds in mango Keitt extract

Effects of Mango Extract A on MCF-12A Net Growth

The net growth of MCF-12A non-cancer breast cells was performed by cell counting after treatment with different concentrations of mango polyphenols (2.5 – 80 µg GAE/ml) for 48 h. The net growth of MCF-12A non-cancer breast cells was inhibited by ~ 50% when the lowest concentration of mango extract A (2.5 µg GAE/ml) was tested. A greater inhibition was observed when higher concentrations of mango extract A were applied to MCF-12A non-cancer breast cells.



Cell proliferation of MCF-12A non-cancer breast cells treated with Mango Extract A. Cells were treated with different concentrations of mango extract and cell growth was assessed after 48 h of incubation. Values are means ± SE (n=3). Different letters indicate significance at $p < 0.05$.